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DENTAL ENAMEL

1997

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Published in 1997 by John Wiley & Sons Ltd,
Baffins Lane, Chichester,
West Sussex PO19 1UD, England

Telephone National 01243 779777

International (+44) 1243 779777

e-mail (for orders and customer service enquiries): cs-books@wiley.co.uk

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John Wiley & Sons, Inc., 605 Third Avenue,
New York, NY 10158-0012, USA

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Ciba Foundation Symposium 205
ix+284 pages, 63 figures, 13 tables

Library of Congress Cataloging-in-Publication Data

Dental enamel.

p. cm. — (Ciba Foundation symposium ; 205)

Editors: Derek Chadwick and Gail Cardew.

Proceedings of the Symposium on Dental Enamel, held at the Ciba Foundation on 23–25 Apr. 1996.

Includes bibliographical references and indexes.

ISBN 0-471-96872-2 (alk. paper)

1. Dental enamel — Physiology — Congresses. I. Chadwick, Derek.
II. Cardew, Gail. III. Symposium on Dental Enamel (1996 : Ciba Foundation) IV. Series.

[DNLN: 1. Dental Enamel — physiology — congresses. 2. Dental Enamel — anatomy & histology — congresses. 3. Dental Enamel Proteins — congresses. 4. Tooth Diseases — congresses. W3 C161F v. 205 1997 / WU 220 D4123 1997]

QP88.6.D455 1997

611'.314 — dc20

DMLM/DLC

for Library on Congress

96-42297

CIP

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN 0 471 96506 5

Typeset in 10/12pt Garamond by Dobbie Typesetting Limited, Tavistock, Devon.

Printed and bound in Great Britain by Biddles Ltd, Guildford

This book is printed on acid-free paper responsibly manufactured from sustainable forestation, for which at least two trees are planted for each one used for paper production.

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Introduction

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National Institute of Dental Research, National Institutes of Health, Building 31, Room 2C39, Bethesda, MD 20892-2290, USA

Dental enamel is at a crossroads. The biological sciences have lagged behind other sciences, such as physics and chemistry, in the large-scale application of advanced technology to research problems. However, over the last 30 years, technology has increasingly demonstrated its potential to catalyse revolutionary breakthroughs in the biological sciences and this is particularly true in the fields of dental enamel — health promotion, disease prevention, diagnostics and therapeutics have been fuelled by the scientific advances in the fundamental basic science of structure and function. In keeping with the philosophy of the Ciba Foundation, ‘promoting excellence in science worldwide’, we have gathered to evaluate strategically what has been accomplished and to identify both new directions for science and technology, as well as health-related opportunities.

Traditionally, we think of scientific discovery as driving the development of technology, but frequently the opposite is in fact the case. Of course, there is a wonderfully close symbiosis between the two intellectual strategies and this cross-disciplinary symbiosis is especially evident in the context of this workshop, with representation from so many different disciplines, technologies and nations of the world.

Advanced and emerging technologies are providing the catalyst for discovery. Imagine what has evolved since 1965. The science of dental enamel has progressed in no small measure from a number of remarkable advances in technology: (i) from the scanning electron microscope to the scanning tunnelling microscope; (ii) from histology to immunocytochemistry; and (iii) from column chromatography for protein isolations and amino acid compositions to human genomic DNA sequencing technology (the lexicon of alternative splicing, promoters, exons, enhancers and introns), PCR techniques, *in situ* hybridization and transgenic animal models to study amelogenesis imperfecta.

The revolution occurring in the biological sciences is evident in the field of dental enamel. Biological information can be deciphered and manipulated at exponentially increasing rates and that is reflected in many of the rapid advances: new multiple genes (e.g. amelogenins, enamelines/tuftelins and ameloblastins) have been discovered for enamel; a number of complementary translational gene products have been isolated and characterized; new technologies are being used to understand the structural

biology of intra- and inter-molecular interactions; new technologies are being used towards understanding structure/function relationships in normal and diseased dental enamel conditions (e.g. antisense inhibition using ribozymes and transgenic animal models); new approaches in cellular, molecular, developmental, evolutionary and computational biology are serving to fuel progress towards understanding the genesis of enamel; and biomimetic or tissue engineering approaches are emerging, which might meet the opportunities for improved diagnostics and a new generation of craniofacial–oral–dental therapeutics.

I would like to acknowledge the key role played by Gerald B. Winter who worked closely with the Ciba Foundation in organizing the workshop and developing its themes, and was instrumental in the realization of this symposium. I would also like to thank the enthusiastic participation of the workshop attendees, who discussed these fascinating issues in a mutually cooperative, efficient and yet intellectually invigorating way. I believe that their efforts reported in the formal presentations and animated discussions have formed a broader focus on emerging scientific and health-related challenges, as well as on new technologies that will provide even more exciting directions in dental enamel in the years to come and well into the next millennium.

Tooth morphogenesis and the differentiation of ameloblasts

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Abstract. All vertebrate organs are formed from several cell types, and it is currently believed that interactions between the different components constitute the most important mechanism in the regulation of organ morphogenesis. In developing teeth morphogenetic interactions occur between the epithelium covering the facial processes and the underlying neural crest-derived mesenchyme. Morphogenesis is accompanied by differentiation of the various dental cell types, including the ameloblasts. Although ameloblasts differentiate terminally and start the deposition of enamel matrix only after the completion of crown morphogenesis, there is increasing evidence suggesting that the segregation of the ameloblast cell lineage may start much earlier. For example, the down-regulation of the Notch receptor, which in some other developmental systems is associated with cell fate determination, is already seen in the dental epithelium prior to the bud stage. It is not known to what extent the differentiation of ameloblasts depends on tooth morphogenesis, and whether the same mesenchymal signals regulate morphogenesis and cell differentiation. There is evidence that growth factors act as morphogenetic signals. Bone morphogenetic proteins and fibroblast growth factors appear to regulate the initiation of tooth development, as well as the morphogenesis of the crown shape. However, the molecular nature of the signals regulating the advancing specialization of the cells in the ameloblast cell lineage remains unknown.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 3–17

Teeth form from the ectoderm covering the facial processes and the underlying mesenchymal cells which derive from the cranial neural crest. The first morphological sign is the thickening of the epithelium, which subsequently buds into the underlying mesenchyme. The mesenchymal cells condense around the bud, and during the following cap and bell stages the epithelium undergoes folding morphogenesis, resulting in the establishment of the form of the tooth crown. These early stages of tooth development are reminiscent of the early morphogenesis of many other organs, such as hairs, glands, kidneys and lungs. Data on the molecular basis of regulation have accumulated with increasing speed during recent years, and the tooth is one of the organs for which plenty of information is available. The current evidence indicates that the similarities between different organs are not restricted to the general

developmental mechanisms, and that the molecular basis of developmental regulation has been conserved to an astonishing extent (Thesleff et al 1995).

The differentiation of the tooth-specific cell types, notably the mesenchymal odontoblasts, cementoblasts and epithelial ameloblasts, is tightly linked to tooth morphogenesis. Although the dental cells do not differentiate terminally earlier than at advanced bell stage (odontoblasts and ameloblasts), or after the completion of crown formation (cementoblasts), there is increasing evidence that their cell fate becomes determined much earlier. It is obvious that the intimate associations between cell differentiation and morphogenesis complicate the independent analysis of the regulation of these developmental phenomena.

Epithelial-mesenchymal interactions

Teeth, like most vertebrate organs, develop from epithelial and mesenchymal tissues. Interactions between the two tissue components, also called secondary inductive interactions, are currently believed to constitute the most important single mechanism in the regulation of morphogenesis. The nature of such epithelial-mesenchymal interactions has been analysed for many decades, mostly in classic tissue recombination studies. The results have indicated that the interactions are sequential and reciprocal in nature and that, according to the stage of development, the tissues may regulate differentiation in more or less instructive ways (Saxén 1977, Thesleff et al 1995).

Experimental tissue recombination studies on developing teeth have established that there is actually a chain of inductive interactions between the epithelium and mesenchyme which regulates both tooth morphogenesis and cell differentiation (Figs 1 and 2). The epithelium acts instructively on the underlying mesenchyme during very early tooth development, and this ability is shifted during the bud stage to the mesenchyme, which subsequently retains the potential to regulate tooth morphogenesis until the bell stage (Mina & Kollar 1987, Lumsden 1988).

Ameloblasts and odontoblasts differentiate during specific stages of tooth morphogenesis, but we do not know at present how greatly their advancing determination and differentiation depends on morphogenesis, and to what extent epithelial-mesenchymal interactions influence the progressive determination and differentiation of these specialized cell types. It has, however, been established in numerous experimental studies that the terminal stages of odontoblast and ameloblast differentiation depend on epithelial-mesenchymal interactions. The terminal differentiation of odontoblasts is triggered by the dental epithelium during early bell stage, and the epithelial basement membrane has been shown to play a central role in this interaction (Thesleff & Hurmerinta 1981, Ruch 1985, Thesleff et al 1995). On the other hand, the pre-dentine matrix deposited by the odontoblasts appears to play an important role in triggering the acquisition of the terminal stage of ameloblast differentiation during the more advanced bell stage (Ruch 1987). It is conceivable that the determination of cells in the ameloblast cell lineage is also

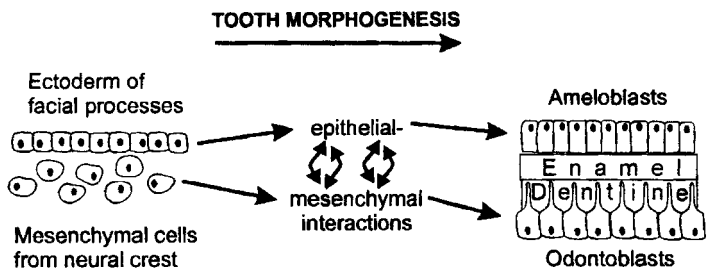


FIG. 1. Schematic representation of the differentiation of the ameloblasts and odontoblasts at the interface between the epithelium and mesenchyme. Ameloblasts derive from the ectoderm covering the facial processes and odontoblasts from the underlying neural crest-derived mesenchymal cells. The progressive differentiation of the ameloblasts and odontoblasts is tightly linked to advancing morphogenesis, during which the epithelial-mesenchymal interface undergoes budding and folding morphogenesis (bud, cap and bell stages). Interactions between the two tissues regulate morphogenesis as well as terminal cell differentiation, which takes place at the bell stage.

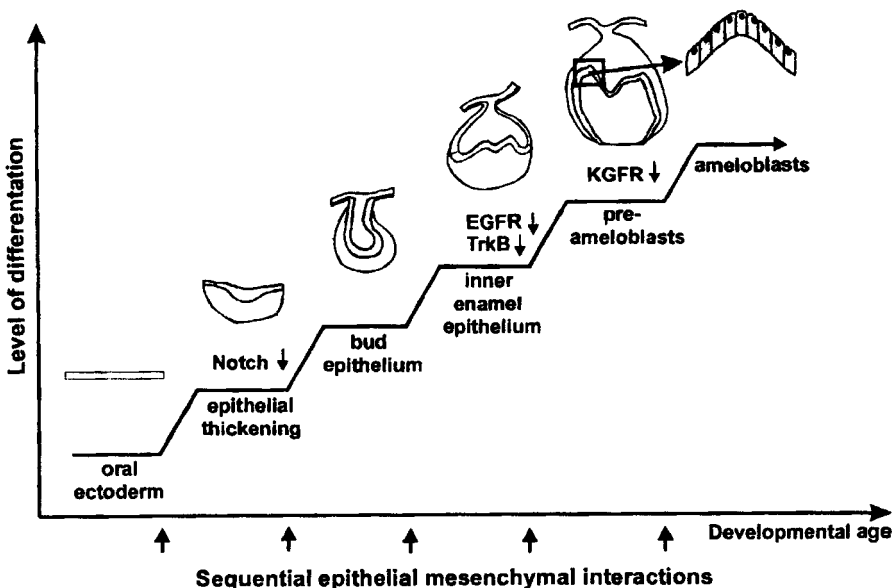


FIG. 2. Advancing specialization of the cells in the ameloblast lineage. The differentiation of the ameloblasts from the cells of the oral ectoderm is tightly linked to tooth morphogenesis, which is regulated by a chain of epithelial-mesenchymal interactions. Several signalling receptors are down-regulated in this cell lineage at various stages of development: Notch receptor prior to bud stage; epidermal growth factor receptor (EGFR) and TrkB at the cap stage; and the fibroblast growth factor receptor KGFR (keratinocyte growth factor receptor) at the bell stage. This may reflect the early acquisition of the ameloblast cell fate.

regulated by mesenchymal signals at earlier stages, at least to some extent. It is possible, however, that mesenchymal factors are not required for all aspects of dental epithelial morphogenesis and differentiation, as was recently shown for the development of the pancreas (Gittes et al 1996).

Changes in gene expression in the ameloblast cell lineage

The progenitors of the ameloblast cell lineage can be traced back to the thickened presumptive dental epithelium, the first morphological sign of initiated tooth development (Figs 1 and 2). During bud stage, these cells form the basal epithelium, which contacts the basement membrane. During the cap stage, the inner enamel epithelium, which faces the dental papilla mesenchyme, becomes discernible from the outer enamel epithelium, which is surrounded by the dental sac mesenchyme. During the bell stage, the inner enamel epithelial cells in the cuspal areas cease to proliferate and subsequently become polarized, columnar pre-ameloblasts. These cells then differentiate terminally into ameloblasts secreting the organic matrix of enamel (Fig. 2).

The advancing specialization or determination of the cells in the ameloblast lineage is evident in changes of the expression of different molecules. Some of the genes encoding structural components of the enamel matrix appear to be already transcribed during the bud and cap stages of development (Couwenhoven & Snead 1994, Zeichner-David et al 1995). This indicates that cell type-specific gene expression is initiated in the ameloblast cell lineage well before the cells become postmitotic and acquire the characteristics of terminally differentiated cells. It is conceivable that regulatory molecules which determine the responses to differentiation signals and regulate the expression of the cell type-specific gene products are expressed in the cells even earlier. Such molecules include transcription factors and signalling receptors. Work in our laboratory has revealed early changes in some cell surface receptors for growth and differentiation factors in the ameloblast cell lineage (see below).

The actions of growth factors and growth factor-like ligands are mediated via binding to specific cell-surface receptors. Hence, in order to respond to the signals, the cells must express the appropriate receptors. It follows that the array of such receptors at the surface of a cell determines its responsiveness to extracellular signals. In the context of inductive interactions, this is termed competence. It is a result of the previous history of the cell and it reflects the state of its determination. We have analysed the expression of several signalling receptors in different growth factor families in developing teeth from the time of initiation of tooth development to the stage of completed crown morphogenesis, i.e. until ameloblasts have terminally differentiated and are secreting enamel matrix. Interestingly, we have observed that the expression of several signalling receptors is down-regulated relatively early in the ameloblast cell lineage (Fig. 2).

The epidermal growth factor receptor (EGFR) was the first receptor to be studied, and was localized by analysing the binding of radioactively labelled EGF to dissected

tooth germs. The EGFR is down-regulated in the inner enamel epithelium at cap stage (Partanen et al 1985). Interestingly, EGF receptors are up-regulated in the ameloblasts again during their maturation (Davideau et al 1995). Our recent *in situ* hybridization analysis has demonstrated that the neurotrophin receptor TrkB, which binds brain-derived neurotrophic factor (BDNF), is down-regulated in the cap stage inner enamel epithelium (Luukko et al 1996). The fibroblast growth factor (FGF) receptor KGFR (keratinocyte growth factor receptor), which binds FGF7, is down-regulated in the pre-ameloblasts at the bell stage (Kettunen et al 1997). As EGF and FGF7 are known to be mitogenic to epithelial cells, their down-regulation may be associated with the cessation of cell proliferation in the pre-ameloblasts.

So far, the most interesting observation is the early down-regulation of the expression of the Notch receptor in the ameloblast cell lineage (Mitsiadis et al 1995). This is a receptor that has been examined intensely in *Drosophila*, and it has been shown to have important roles in cell fate specification. Interestingly, loss of Notch activity leads to an excess of neuroblasts at the expense of epidermal cells. Delta and Serrate are cell surface-bound proteins in neighbouring cells that have been identified as ligands for Notch in *Drosophila* (Artavanis-Tsakonas et al 1995).

We analysed the expression of all known vertebrate Notch genes (*Notch 1, 2 and 3*) in mouse teeth, and showed that each of them is down-regulated in the basal cells of the thickened presumptive dental epithelium (Fig. 2). This down-regulation persists in the ameloblast cell lineage throughout its progressive differentiation during the bud, cap and bell stages, whereas all other dental epithelial cells (stratum intermedium, stellate reticulum and outer enamel epithelium) express the Notch genes intensely (Fig. 3). This down-regulation of the Notch genes is at present the earliest molecular change observed in the ameloblast cell lineage.

We also studied the regulation of *Notch* expression in organ culture experiments. In explants of recombined dental epithelium and mesenchyme, *Notch* expression was down-regulated in dental epithelium juxtaposed to mesenchyme, indicating that the dental epithelium needs a mesenchyme-derived signal in order to maintain down-regulation of *Notch* (Mitsiadis et al 1995). Hence, this suggests that epithelial–mesenchymal interactions may regulate differentiation in the ameloblast cell lineage at very early stages of morphogenesis.

Growth factors as inductive signals

The molecular nature of the signals that mediate inductive cell and tissue interactions began to be elucidated in the late 1980s. The first evidence that growth factors may act as inductive signals in vertebrate embryogenesis came from studies on mesoderm induction (Slack 1994). Subsequently, expression of growth factors and their receptors has been localized in various developing organs, specifically at sites where epithelial–mesenchymal interactions were known to take place (Lyons et al 1991, Pelton et al 1991). Also, during tooth development a number of growth factors and receptors have been localized at the correct places and times, suggesting functions in

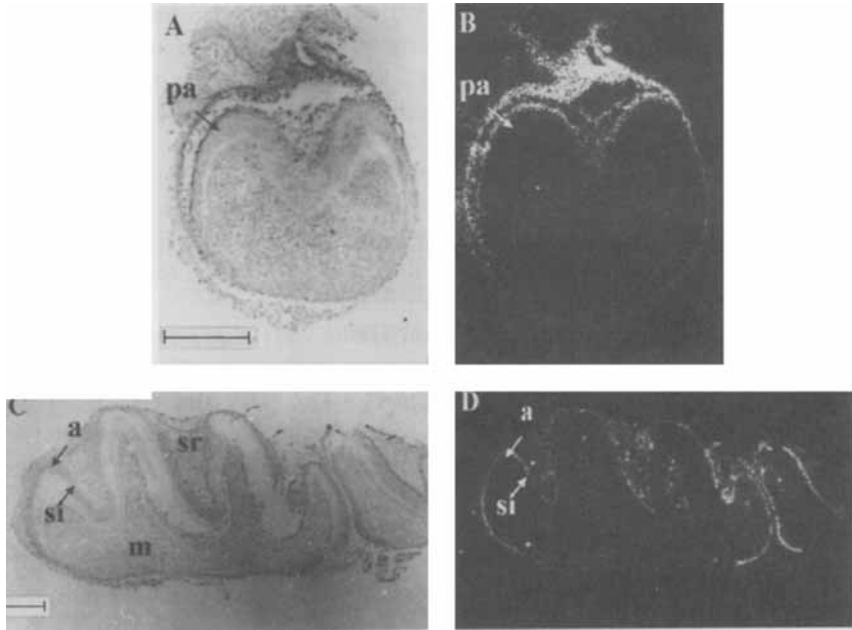


FIG. 3. Down-regulation of the expression of *Notch* signalling receptors in the ameloblast cell lineage. *In situ* hybridization analysis of sections of embryonic day 19 (A, B = darkfield image of A) and postnatal day 6 (C, D = darkfield image of C) mouse molar tooth germs. Neither *Notch 2* (A and B) nor *Notch 1* (C and D) are expressed in the ameloblast lineage (arrows), whereas other epithelial cells show intense expression. Down-regulation becomes evident in the ameloblast cell lineage prior to bud stage of development (Mitsiadis et al 1995). a, ameloblasts; m, dental mesenchyme; pa, pre-ameloblasts; si, stratum intermedium; sr, stellate reticulum (figure courtesy of T. Mitsiadis). Bars = 100 μ m.

inductive signalling (Thesleff et al 1995, Fig. 4). Results from our laboratory have indicated that two members of the bone morphogenetic protein family, BMP2 and BMP4, may function as epithelial signals during early tooth morphogenesis (Vainio et al 1993). *Bmp4* is expressed in the thickened presumptive dental epithelium and its expression shifts to the mesenchyme during bud stage (Fig. 4). Thus, its expression associates with the shift of odontogenic potential from the epithelium to the mesenchyme. *Bmp2* is expressed in the dental epithelium at the time of budding (Åberg et al 1997). *Bmp2* and *Bmp4* recombinant proteins mimic some of the effects of the dental epithelium on the mesenchyme when applied locally in agarose beads on dental mesenchyme in culture. Most interestingly, they induce the expression of the homeobox-containing transcription factor *Msx1*, which *in vivo* becomes intensely expressed in the condensing dental mesenchyme at bud stage (Vainio et al 1993). Transgenic mice lacking functional *Msx1* develop no teeth, thus showing the necessity of this molecule for tooth morphogenesis (Satokata & Maas 1994).

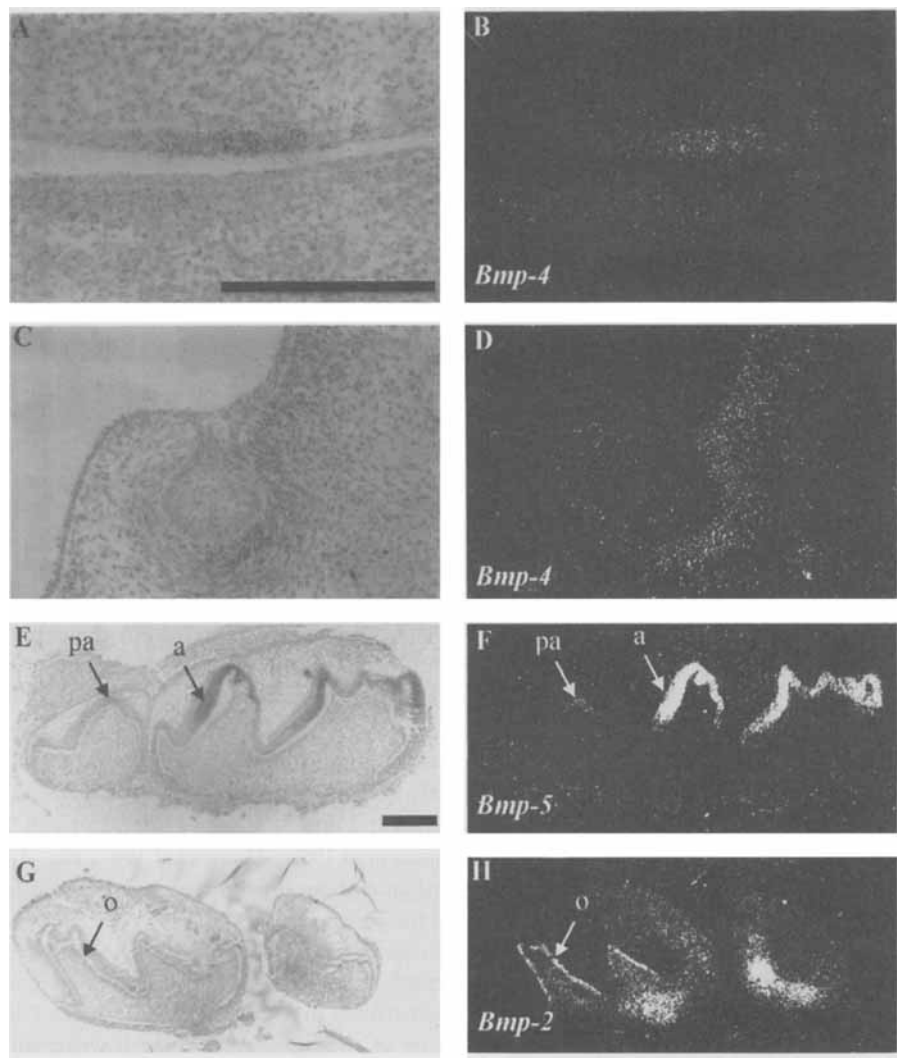


FIG. 4. Expression of bone morphogenetic protein (*Bmp*) transcripts in the epithelial and mesenchymal cells at the tissue interface suggests signalling roles for these growth factors (*in situ* hybridization analysis). (A and B) *Bmp4* is expressed in the early dental epithelium during initiation of tooth development, and (C and D) in the underlying dental mesenchyme during bud stage. (E and F) *Bmp5* is expressed by pre-ameloblasts and ameloblasts (arrows; a, ameloblast; pa, pre-ameloblast). (G and H) *Bmp2* is intensely expressed in the odontoblasts (arrow; o, odontoblast). Bar in A = 100 μ m (applicable to A–D). Bar in E = 100 μ m (applicable to E–H).

Our recent computer-based 3D analysis of the expression patterns of several growth factors has revealed the transient existence of an actual signalling centre in the tooth germ. This centre resides in the enamel knot, a cluster of dental epithelial cells that becomes morphologically evident during the cap stage. However, gene expression data indicate that the enamel knot is already formed at the bud stage (Jernvall et al 1994, Vaahtokari et al 1996). Cells in the enamel knot express three different *Bmps* (*Bmp2*, 4 and 7) as well as *Fgf4* and *sonic hedgehog*. We do not know whether the enamel knot-derived growth factors signal to the mesenchyme or to the adjacent epithelium, or to both, but we believe that the enamel knot functions as an organizing centre, regulating the morphogenesis and growth of tooth cusps. It is remarkable that the same signalling molecules are expressed in several organizing tissues in the embryo, including the notochord, the zone of polarizing activity and the apical ectodermal ridge in limb buds (Roelink et al 1994, Tickle 1995). This suggests that the signals regulate some conserved aspects of morphogenesis and pattern formation.

There is evidence that the BMP family of growth factors are potent inducers of terminal odontoblast differentiation. BMPs evoke a response of reparative dentine formation when implanted in the tooth pulp (Rutherford et al 1993). *In vitro*, BMPs stimulate the differentiation of embryonic dental papilla cells into odontoblasts (Beque-Kirn et al 1992). Several *Bmps* (*Bmp2*, 4, 5 and 7) are expressed in the dental epithelium at various stages of tooth development (Vainio et al 1993, Åberg et al 1997, Fig. 4), so it is possible that BMPs also regulate odontoblast differentiation *in vivo*.

Evidence for possible roles of growth factors in ameloblast differentiation is still lacking. The newly differentiated odontoblasts express many growth factor mRNAs, including transforming growth factor β (*Tgf β*) 1, 2 and 3, as well as *Bmp2*, 4 and 7 (Pelton et al 1991, Vaahtokari et al 1991, Vainio et al 1993, Åberg et al 1997, Fig. 4), and as this correlates with the time of terminal ameloblast differentiation it is possible that these growth factors act as mesenchymal inductive signals.

The general theme which is emerging is that the same BMPs and other growth factors are used repeatedly for morphogenetic signalling in different embryonic organs as well as for the regulation of differentiation in a variety of cell types. The current knowledge on the upstream regulators of the signals and their downstream targets is still very limited. There is evidence, especially in *Drosophila*, that various growth factors regulate the expression of homeobox-containing and other transcription factors (Thuringer & Bienz 1993). Also, in vertebrate organs homeobox-containing genes appear to be targets of growth factors, as shown in limb development (Tickle 1995, Niswander & Martin 1993) and in the tooth, where BMPs regulate the expression of *Msx1* and *Msx2* (Vainio et al 1993). It is also noteworthy that the *TGF β* family of growth factors and the BMPs affect the deposition of extracellular matrices in several ways (Adams & Watt 1993). Hence, it is possible that these growth factors regulate the formation of dentine and enamel matrices in the differentiating odontoblasts and/or ameloblasts.

Acknowledgement

This work was supported by the Academy of Finland.

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DISCUSSION

Snead: You mentioned that *Notch* is expressed in stratum intermedium cells during ameloblast differentiation. Does it play a role in the final determination of the ameloblast phenotype in terms of promoting the signalling between the stratum intermedium and the developing ameloblasts?

Thesleff: This is an interesting question. No one has done any experimental studies of this, but the mapping of the Notch ligands, such as Delta and Serrate, may give us some clues because when we know where the ligands are expressed, we may be able to learn something about the interactions (T. Mitsiadis, unpublished results 1995).

Rosenbloom: Are there any unique markers in the stratum intermedium that can be used to identify these cells specifically?

Diekwisch: Certain alkaline phosphatase epitopes are expressed at a high level in cells of the stratum intermedium and not in the ameloblasts.

Zeichner-David: But this depends on the concentration and the method used. There are no significant differences in the levels of expression of alkaline phosphatase in the

cells of the stratum intermedium and the ameloblasts, and there are no current markers for the stratum intermedium.

I would like to ask Irma Thesleff to speculate what bone morphogenetic protein (BMP) 5 and BMP2 are doing in differentiated ameloblasts.

Thesleff: It is possible that they are involved in the regulation of secretion.

Zeichner-David: Have any regulatory roles been assigned to these specific factors in other tissues?

Thesleff: It is known that in the bone system the BMPs have an inductive effect on the formation of bone.

Sharpe: BMP11 and BMP12, which are also known as growth differentiation factors, have not been found in teeth. They are specific to joint formation.

Nanci: What is the difference between the enamel cord and the enamel knot?

Thesleff: The enamel cord is an epithelial structure that links the enamel knot to the outer enamel epithelium. *Msx2* is expressed both in the enamel cord and in the enamel knot, but expression of the different signals is limited to the enamel knot.

Sharpe: It appears that the expression of *Msx2* in the region of epithelial thickening was maintained in the enamel knot, giving the impression that they are the same cells. What happens to *sonic hedgehog* expression after epithelial thickening? Does it disappear?

Thesleff: The expression of *sonic hedgehog* disappears from the budding epithelium in the molars.

Sharpe: Does it have the same expression patterns in the incisors?

Thesleff: We haven't looked very carefully, but there do seem to be differences between its early expression in incisors and molars.

Sharpe: We have found that prior to budding its expression is more pronounced in the incisor region but we do observe some expression in the molar region as well, which contrasts with what has been reported by Kronmiller et al (1995).

Snead: Are there differences in the techniques you are using? Irma Thesleff is using section *in situ* hybridization.

Sharpe: We are using whole-mount *in situ*, so this is a possible explanation.

Rosenbloom: There are numerous BMPs but if they all operate in a common pathway, how is it possible to maintain specificity? Does each BMP have a different receptor, and do these receptors behave differently?

Thesleff: Four tyrosine kinase receptors have been identified for the fibroblast growth factors (FGFs), and it is known that different ligands may use the same receptors and vice versa. The BMPs use serine-threonine kinase receptors, and, furthermore, interaction is required between the type 1 and type 2 receptors, which dimerize.

Rosenbloom: But the cell surface receptors used by the BMPs are all similar to those used by transforming growth factor β (TGF β), so how is specificity achieved?

Thesleff: It is possible that specificity is not required — BMP2, BMP4 and BMP7 may all have identical effects.

Rosenbloom: So they may be redundant.

Thesleff: Yes. It is possible that many BMPs have evolved which are expressed in different places and have differences in their promoter regions so that they are

regulated differently. It is also possible that the growth factors have similar effects. This could be studied experimentally, by expressing constructs where the promoter regions are switched.

Butler: What have knockouts of the BMP genes told us about their roles in tooth development?

Thesleff: Very little. The *Bmp7* knockout is one of the only knockouts that doesn't die early. These mice have a number of congenital defects but no dental defects have been reported (Dudley 1995, Luo et al 1995).

Snead: The *Bmp7* knockout looks very much like the *Msx2* overexpressing mouse. This overlap in phenotypes suggests a common signalling pathway and some degree of redundancy.

It must be difficult to excise, extirpate and move the enamel knot. How are these experiments progressing? Because they are very powerful experiments.

Thesleff: It is relatively easy to excise the enamel knot, but it is more difficult to put it back. For example, we have tried to transplant a molar enamel knot into an incisor, but as the knot is composed of epithelial cells, we try to insert it in an incision of the incisor dental epithelium, which proliferates in response to tissue injury after excision. Therefore, when the knot is put back, it doesn't readily stay within the epithelium, and the resulting tooth shape is difficult to analyse.

Snead: What is the wounding response of dental epithelium?

Thesleff: The structure becomes folded, so that in culture a structure is produced that resembles cusps anyway.

Diekwisch: What is your opinion of the nerve induction theory of tooth bud formation? Neural crest cells migrate into this area, so could nerve-like structures have an early or late role in the induction processes that you have described?

Thesleff: We do not have any evidence that nerve-like structures have a role in the initiating events, but we have performed some mapping studies of the different neurotrophin receptors and neurotrophins (Luukko et al 1996), and we have found some clear correlations between their expression and morphogenesis, so I believe that they could have a role later on.

Slavkin: You showed that these molecules are expressed at an informative stage of limb development, and they are also expressed during lung development and breast development. What is your opinion of combinatorial decisions that are involved in these processes? Can the same molecules be put together in unique combinations to provide the instructions for specificity? Have people approached this problem with multiple knockouts?

Thesleff: As far as I am aware, these experiments have not been done. Specificity may arise as a result of different combinations of signals and transcription factors. Alternatively, specific regulatory molecules may be involved, although there is no evidence of any tooth-specific regulatory genes so far.

Slavkin: You stated that we have learned very little from the knockout experiments. However, the *Msx1* knockout mouse did not develop molars but did develop primitive incisors. This evidence suggests that there are a number of variables, such

as morphogenetic gradients of morphogens, and also different signal pathways that influence the cell cycle.

Thesleff: Yes, but it's also possible that different molecules are present in incisors and molars.

Winter: During differentiation there must be a more supreme control mechanism involving homeobox genes than the one involving inductive effects. How many homeobox genes are involved in this process?

Thesleff: The homeobox genes will probably turn out to be important in terms of the combinatorial idea. Paul Sharpe has speculated on the different combinations of homeobox genes involved in this process, so perhaps he would like to comment on this.

Sharpe: In the model that we have proposed, for which we have evidence that at least part of it is correct, there are genes in signalling systems which are active in all teeth regardless of their shape or position, and they may also be active in other organs. In addition, there are tooth-specific systems that operate independently. For example, certain homeobox gene knockouts just lack molars, and the rest of the teeth are completely normal. Therefore, there are different levels of control: those that are involved in making the tooth and others that determine which particular tooth is going to form in which particular position. My belief is that homeobox genes are central players in the second level.

Snead: One fly in the ointment might be the ectopic overexpression data of Fuchs & Zhou (1995), who showed that in contrast to the *Lef1* knockouts of VanGenderen et al (1994), which fail to develop teeth, the overexpression of *Lef1*, driven by the K1 promoter, results in the production of supernumerary teeth. These supernumerary teeth go through the same pattern of development, suggesting that a single triggering mechanism is capable of re-inducing the entire spectrum of signals and cascades triggered by the LEF1 molecule. It also suggests that in these transgenic lines all of the epithelium in the oral cavity is competent to make teeth. However, it describes competency at the level that most developmental biologists would have some chagrin with in trying to understand because ectopic overexpression of *Lef1* results in the development of supernumerary teeth that are all in the incisor form. It's not clear whether root formation is also induced.

Rosenbloom: What is known about the downstream genes that are responsive to homeobox genes such as *Msx1*?

Sharpe: Not much is known in terms of tooth development. However, these genes also function in osteoblasts during bone formation. We have evidence that *Msx2*, for example, down-regulates osteocalcin, and that *Msx2* up-regulates the expression of alkaline phosphatase.

Snead: Co-transfection experiments have also shown that *Msx2* also down-regulates the expression of amelogenin.

Deutsch: Is there any evidence of apoptosis in developing ameloblasts?

Thesleff: Yes, there is evidence. Smith & Warshawsky (1977) concluded that ameloblasts die on the basis of quantitative analysis of cell turnover. We recently

localized apoptosis in mouse molar ameloblasts by Tunel staining (Vaahtokari et al 1996).

Deutsch: Is anything known about the signals that regulate this?

Thesleff: Not as far as I am aware.

Slavkin: Before the term 'apoptosis' became a dominant scientific problem area in cellular, molecular and developmental biology, Charles Smith showed in his PhD thesis investigations at McGill University in Montreal (circa early 1970s) that a significant number of cells within the enamel organ epithelia of developing rat incisors die. As I recall, this PhD thesis was the first study to identify and map in three dimensions when and where specific sets of enamel epithelial cells die. It would be potentially important to revisit Charles Smith's studies using currently available biomarkers to identify initial apoptosis and thereby to define putative clusters of cells in a particular cell lineage within the enamel organ.

Boyde: It could have a critical role at the secretory-maturation transition stage because many cells die at that stage.

Nanci: This is actually the stage at which Charlie Smith performed his analyses. Smith & Warshawsky (1975) found that 25% of ameloblasts die as they enter the maturation stage, and another 25% die during maturation.

Slavkin: I envision that as the proliferating inner enamel epithelia become postmitotic along the gradient of epithelial differentiation, the signal pathways which control stability or inhibit proliferation are switched to a programmed cell death pathway. This would seem to provide an excellent experimental opportunity to identify the molecular controls for various stages of the presumably multiple cell lineages and cell fates within the inner enamel epithelia; for example, to test the hypothesis that there are two lineages: (1) presecretory, secretory and postsecretory ameloblasts; and (2) another cell lineage which includes time- and position-specific apoptosis.

Snead: As the tooth emerges and the secretory ameloblasts subside, the entire enamel organ collapses on itself. Are any of the cells that remain in the enamel organ transformed?

Diekwisch: There have been studies of the expression of keratin filaments in the stellate reticulum, but is not clear whether a transformation takes place.

Slavkin: I have assumed that the stages of embryonic mouse molar tooth development that Irma Thesleff has described, and in particular the enamel knot signalling molecules, perhaps provide a model for studies designed to test the hypothesis that the enamel knot is a specific region from which a subpopulation of enamel organ epithelia 'transdifferentiate' into various epithelial phenotypes, such as the stratum intermedium, stellate reticulum and/or apoptosis.

Sharpe: One should be careful in saying that they may be derived from the enamel knot. Irma Thesleff has evidence that the enamel knot disappears via apoptosis, and indeed it has to disappear in order for the cusps to form. However, this is not to say that signals derived from the enamel knot initiate the differentiation of other epithelial cells.

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Microstructure of enamel

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Abstract. Enamel is a composite material consisting of mineral and organic phases. The properties of the mineral phase are modulated dramatically by its division into microscopic crystals, cemented together by the organic matrix protein polymer. A good concept of the 3D orientations of the crystals derives from visualizing their growth perpendicular to the surface in which they develop, which is pitted by the secretory poles of the ameloblasts. The arrangement of the crystals is the cause of the discontinuities, known as the prism boundaries or junctions, in the otherwise continuous structure. These locations acquire a more concentrated organic matrix during maturation, and they are both crack stoppers and crack propagation tracks in the adult tissue. Any tendency of prisms to cleave may be reduced by their varicosities, which reflect daily variations in the rate of production; their cross-sectional shape; the non-parallelism of adjacent groups, which develops through translocation of groups of cells across the surface during development; and the support of any one microscopic tissue element by other tissue, including dentine, placed to resist an applied load. Incremental growth lines are preferential cleavage planes within the enamel. Failure patterns of enamel in normal and abnormal use can be explained by these parameters, with additional consideration of functional variation and fatigue.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 18–31

Enamel has a beautiful microstructure. It is tender when first secreted and extremely well protected from mechanical disturbance in its earliest days by the stellate reticulum of the enamel organ. It becomes tough before it begins to function, its strength being reinforced by the dentine mould upon which it is cast and, in many herbivorous mammals, by further support from cementum, which is a form of bone.

Crystallites

The earliest-formed elements to survive into the fully mature adult structure are the central parts of the crystallites (Boyde 1964). These crystals grow first in length and later in width. Young enamel contains only the initial, central portions of the adult enamel crystals, which have a higher magnesium and carbonate content than their peripheries (Boyde 1979). As the crystals thicken, the amelogenin-rich matrix in which they once grew is resorbed by the secretory ameloblasts. The rate of

thickening varies considerably between mammalian species: those in which the teeth last longer grow more mineral at an earlier stage of development (i.e. the crystals are fatter, the water and protein contents are less, and the tissue is harder sooner). Longer-surviving enamel usually also has a longer maturation history, enabling it to become harder.

Groups

The long, crystallographic c -axes of crystals within given microscopic domains grow nearly but not quite parallel to each other. Their flattened hexagonal cross-sections may be aligned such that the larger sides are parallel, and they are thus clustered into groups. They can be seen more easily in younger enamel, which is easier to section for transmission electron microscopy. If these groups survived in mature tissue, they would be the size of the seemingly single crystals observed in low resolution scanning electron microscope images of acid-etched enamel.

Interpit continuum

If it were not for the complex shape of the developing enamel surface (i.e. the interface between the secretory ameloblasts and what they have secreted), then all the c -axes would be parallel and perpendicular to the surface. Where the rate of accretion is very low (e.g. $<1 \mu\text{m/day}$) the interface tends to be nearly flat and there is little modulation of the underlying crystal orientation.

However, the most common situation in mammals is that the secretory product is released from the cell at two preferred sites. The more superficial site forms the most prominent parts of the entire developing enamel surface, where the enamel matrix is released just below the level of the continuous belt of intercellular attachments. This site is interameloblastic and the product defines pits, one of which relates to each cell. It is convenient to adopt the name interpit for this phase (this being developmentally correct, and bypassing the errors and confusion resulting from the usual nomenclature based upon the interpretation of the adult tissue structure). In general, the interpit phase is continuous, without any identifiable phase boundary, throughout vast regions of the tissue. It can occur as: (1) an interprismatic phase in pattern 1 enamel (that having more or less circular or hexagonal prisms); (2) the phase that can be conceived to lie in the same locations even though the dividing lines of the prism junctions or boundary discontinuities are incomplete, as in pattern 3 enamel (Fig. 1A); or (3) the longitudinal inter-row sheets of interprismatic enamel in pattern 2 enamel (Fig. 1C; Boyde 1964, 1989). Furthermore, the long crystal axes are perpendicular to the general plane of the developing enamel surface.

The second location at which enamel matrix is released is from the secretory pole ('Tomes') process proper, which serves to fill in the pit. Crystals forming at these sites may have orientations that merge with crystals formed in the interpit phase, forming open-sided prism boundaries. This occurs in the classical pattern 3 arrangements, such

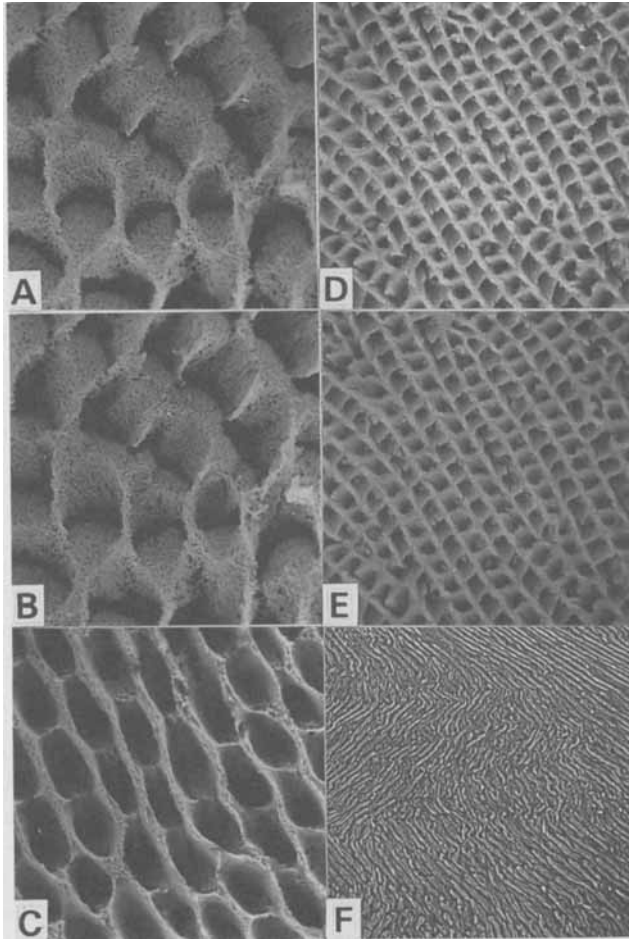


FIG. 1. (A & B) Stereo-pair 10 kV scanning electron microscope image of freeze-dried, anorganic developing human (pattern 3) deciduous enamel surface showing instances of two and three prism boundaries joined in arcades (Fieldwidth = $23\text{ }\mu\text{m}$). (C) 10 kV scanning electron microscope image of critical point-dried, plasma-ashed developing camel (*Camelus dromedarius*) deciduous molar enamel surface showing pattern 2 enamel formation (Fieldwidth = $35\text{ }\mu\text{m}$). (D & E) Stereo-pair 10 kV scanning electron microscope image of critical point-dried, osmium-fixed developing dormouse (*Glis glis*) incisor inner enamel surface showing transverse rows of Tomes' process pits with alternate exit directions associated with underlying decussating prisms (Fieldwidth = $81\text{ }\mu\text{m}$). (F) 20 kV backscattered electron-scanning electron microscope image of etched white rhinoceros (*Ceratotherium simum*) molar enamel (surface cut longitudinally) showing mutual border of vertically decussating zones (Fieldwidth = $187\text{ }\mu\text{m}$).

as in the majority of human enamel. If the 'Tomes' process pits are not very deep, then we can identify a 'floor' to the pit, where the crystals are nearly perpendicular to that floor. Since the floor may be continuous with the wall of the pit on one side, the floor must be tilted and the crystals below will have an orientation different from those of the interpit phase.

Except in the case of some pattern 1 enamels, where pit floors may be nearly perpendicular to the mean plane of the developing enamel surface and the associated crystals therefore also nearly perpendicular to the surface, there are substantial differences in the mean orientations of pit wall and pit floor crystal groups, and where these meet, the structure is discontinuous. (This boundary is commonly called the prism sheath, but the priority in this term lies with the structure developing to either side of this plane upon acid 'etching': Boyde 1990.) In the case of deep pits, the erstwhile floor may be highly tilted: crystals are then best described as lying in the net direction of progress of the ameloblast with time, i.e. the prism direction.

Nature of the orientation discontinuity

The orientation discontinuity is mostly of the nature of crystals crossing each other. This is easily understood both in a strongly developed pattern 2 arrangement, where the crystals in the inter-row sheets cross those within the essentially parallel bundles or prisms, and around the greater part of a pattern 3 boundary. In the latter case, however, there is a second class of orientation change where one set ends obliquely head-on to the sides of the other, or sets clash obliquely head-on. A third aspect prevails where there is little, if any, difference in the orientation of adjacent domains, but their different developmental timing and location determines that there is a locally different packing density: the concentration of protein in the orientation discontinuity plane does not differ at the initial secretory step, but increases during the maturational history of the tissue.

An additional misorientation seam, corresponding to the presence of an additional surface concavity in the sloping floor of the Tomes' process pit, is seen in bat enamel development (Lester & Boyde 1987).

Cross-striations and varicosities

Since new enamel matrix is released from those sides of the Tomes' process at which we can deduce that there is least relative sliding motion, Boyde (1964) hypothesized that the proportions of matrix released closest to (interpit) and furthest from the cell body (pit floor) might relate to the rate of secretion (less of the former when faster), which might vary in a circadian rhythm. This model predicted the observed form of the sinuous outline of the prism boundary, giving rise to an element of varicosity of the enamel prisms. Such features occur with the same periodicity as changes in the 'density' or composition which can be seen in backscattered electron (BSE)-scanning electron microscope images (Boyde 1979, Boyde & Jones 1983). This may reflect a variation in

the net composition of the mineral component (Mg^+CO_3 vs. Ca^+PO_4). This proposition would demand that a significantly high proportion of the width of the enamel crystal be formed soon after its initial propagation into new matrix, and that enamels with high initial mineralization rates should show more marked variations in mineral composition. The data available to date suggest that this is true.

Micro- and macro-decussation

Ameloblasts move both away from dentine in a perpendicular (+Z) direction in the course of time and parallel to the surface in the XY plane. The translocation or migration of ameloblasts across the surface that they fabricate explains the phenomenon of decussation of the formed elements in the enamel.

In pattern 2 enamel formation (Fig. 1C) the cells move vertically (+Y), giving rise to prisms that make a low angle of incidence to the mean plane of the developing enamel surface. Meanwhile, the inter-row sheet enamel is formed between the adjacent longitudinal rows of cells, a phase containing essentially surface-normal crystals. The prismatic bundles of crystals form partially interrupted sheets running close to the vertical axis (+Y) of the tooth which decussate with inter-row sheets (+Z), often nearly at 90° . The shared origin of the interpit and inter-row sheet phases can be further understood from this analysis: not only is this phase shared between cells, but it is also formed between cooperating sheets of cells. The same inter-row sheet crystallite may grow through matrix made by many different cells, albeit of the same local longitudinal lineage.

In sciuriform and myomorph rodent incisor inner enamel formation, alternate single transverse rows of cells move in opposite directions (Fig. 1D & E). The resultant prismatic bundles form sheets that decussate. The interpit crystals do their best in filling in the space in this structure. Depending upon the vertical (incisal) component of movement—which determines the incisal inclination of the prisms—the interpit crystals may weave through the intervening space to produce a 3D interlocking structure with, for example, two sets of domains at right angles and a third at 45° to the common plane of the other two sets (Boyde 1964, 1978a, Kallenbach 1973).

The two examples just cited illustrate both a general principle and some extremes. Namely, we see the mini-decussation of pit-outlining versus pit-infilling components, and the macro-decussation of zones of prisms giving rise to the Hunter-Schreger bands of enamel, containing prisms having common but contrasting orientation properties. The first, local type implies movement of the cell with respect to that part of the matrix which it first assembled by the time that the second is released. The second involves movements of cells relative to each other within the plane of the sheet. In neither case has it been determined which mechanisms control the intricate behavioural patterns: they are clearly under genetic control. Family and even species identification may be gleaned from the study of enamel microstructure (Tomes 1849,

1850, Korvenkontio 1934–1935) and, because it is the only tissue formed as a near-fossil, enamel excites great interest from palaeomammalogists.

Hunter–Schreger bands are usually +X–X movement related, i.e. cells move laterally, left or right, parallel with the differentiating edge of the tooth germ and parallel with neighbouring cells from their own developmental horizon. In rhinoceroses the sense of decussation is rotated through 90° such that cell columns move in the +Y–Y sense, perpendicular to the local developmental horizon (Boyde & Fortelius 1986). Clear evidence of local vortices (analogous to maypole dancing patterns: +X+Y–X–Y) can be seen both in the first enamel to form on dentine at cusp tips, and at the border of decussating zones (Fig. 1F; Kawai 1955, Boyde & Fortelius 1986).

Incremental features

The formative life history of a tooth and of teeth in a dentition can be traced from the study of variations in enamel quality and quantity, which reflect unknown systemic influences that interplay with a varying sensitivity of the ameloblasts in different (developmental) regions of a tooth to such generalized (patho-) physiological rhythms or disturbances.

In human enamel a periodic emphasis of the regular cross-striations occurs at a frequency of about eight to 10 (nearly a week, circaseptan incremental lines). There is a change in mineral content/composition, as evidenced by BSE imaging, and a two-step change in the position of the prism boundary discontinuity: the prism becomes narrower due to the increased amount of interpit phase material, which determines that the prism boundary inclines inwards resulting in an orientation with an enhanced component lying in the plane of the incremental surface; and secondly, the original prevailing situation recovers (Risnes 1990).

At lower lateral and cervical levels in human permanent teeth, where the enamel formation rate—judged from the cross-striation repeat interval—is reduced, the prism boundary markings may be seriously disturbed. Sometimes there is evidence for a defect in crystallite continuity across the incremental line. The plane then appears as a continuous fault. Elsewise, and particularly in immediately subsurface tissue, the boundary discontinuities may disappear only to reform in later tissue. Finally, we may see the formation of non-prismatic enamel, yet with clear circadian interval lines of narrow spacing that can be traced back in continuity into the deeper tissue with its more widely spaced lines.

At the finished surface, the perikymata, or imbrication lines, are manifestations of the internal layering. They show a gradient along the tooth surface. At earlier (more incisal or occlusal) levels, there are bands that display pits. These often have prism boundary discontinuities projecting to the very surface and are followed by bands where the enamel secretory ability has recovered, permitting the formation of non-prismatic and nearly smooth-surfaced tissues. In the latest horizons on any one tooth, the surface incremental phenomena show as cut-offs in the formation of non-prismatic

tissue, with a corresponding reduction in the height of the next imbricating layer (i.e. because the daily formation rate is low, the height of the cliff representing its deficiency is also low).

Start-up zone features

The union of enamel with dentine is intimate, there being no hard and fast dividing plane between the two. The matrix of enamel intermeshes into the surface of dentine: crystals at the junction might have originated in one matrix or the other and intermediate crystal morphologies may exist. Even at low magnification resolution, headlands of dentine project into enamel.

Enamel tubules are features equivalent to dentine tubules and canaliculi in bone, having once been occupied by processes of ameloblasts that communicated with odontoblasts (Tomes 1849, Lester et al 1986). In humans they are numerous, but they are also short and narrow, and so usually escape detection by routine low magnification methods. They extend for great distances into enamel in most marsupials (and some insectivores and lemurs).

In the giant fossil macropod *Diprotodon* the enamel tubules end in dilated extensions strongly reminiscent of the remaining communicating feature at the dentine–enamel junction, i.e. the spindle (K. S. Lester, personal communication 1988). The form suggests that the associated ameloblast died and was encapsulated as a space in enamel before significant autolytic destruction could occur.

Functional aspects of enamel structure

The structural organization of enamel may be such as to enhance hardness and wear resistance. Alternatively, it may have no special functional meaning or it may predispose to tissue loss to secure some special advantage for a particular functional requirement.

The parallel arrangement of crystals perpendicular to the tooth surface gives rise to the best chance for dense packing of crystals and obviates the need to nucleate more than a minimal number of new crystals during development (since they grow at their free ends into any newly available matrix). It is microporous, so that extra mineral may be allowed in to enable the crystals to grow and the degraded matrix components may be removed, leaving the upgraded matrix to adapt to its function as intercrystalline glue and as the continuous phase of a fibre-reinforced composite material.

The perpendicular orientation also allows the growth of long whisker-like crystals for maximum single crystal strength and bendability: single crystals would be too flexible. Their cementing together into groups and larger domains enables them to be stiffer and stronger.

When enamel cleaves through parallel crystal domains, it does so by following the crystal orientation where possible, evidence that the break occurs within the matrix glue rather than within the crystals. Wherever possible, however, cracking seems to

take the line of least resistance, following the existing discontinuities, i.e. the prism boundaries and the incremental lines.

A structure made of parallel fibrous elements, given that the fibres (either crystals, groups of crystals, prisms or inter-row sheets) are stronger than the intervening matrix holding them together, will wear more rapidly where the long axes are parallel to the wear surface (Boyde 1983). Thus, the initial orientation of both crystals and prisms normal to the unworn incisal or occlusal tooth surface is the best to secure wear resistance. However, a worn tooth could hardly continue to satisfy this simple criterion.

The (pattern 2) enamel found in the great majority of herbivorous mammals is subject to a high rate of wear. The prism bundles are more nearly normal to the wear surface: the crystals in the inter-row sheets are parallel to the wear surface, but there are no domain discontinuities in the inter-row sheets. Thus, most existing discontinuities (and any may be exploited in increasing wear rate) are perpendicular to the wear surface.

Rhinoceroses are, or they have evolved from, browsers. The near vertical decussation of the Hunter-Schreger bands in the inner part of the enamel (which is pattern 3) gives rise to unequal wear rates, with surface parallel zones lost preferentially. This causes a microscopic serration of the functional surface of the inner enamel, which presumably has a functional advantage in cutting tougher vegetation such as the harder stems of leaves and twigs (Boyde & Fortelius 1986).

Human teeth wear such that prisms are either nearly perpendicular or more nearly parallel to functional facets. Consider, as a simple example, an upper incisor with normal occlusion. By the time the dentine is exposed, the labial enamel wears roughly parallel with the prisms, so that the labial, cutting edge is sharp. The palatal side of the worn edge, which merges smoothly on to the palatal face, is more worn but also more wear resistant (the situation is the mirror opposite for the lower incisors).

Developmental faulting occurs prior to full maturation, probably to release in-built strain resulting from an internal swelling pressure due to the ongoing crystal growth. Tufts develop where enamel matrix proteins migrate to fill in the faulting voids, which therefore contain reduced mineral and enhanced matrix concentrations. The direction followed by this early developmental faulting (and the later faulting leading to the formation of lamellae) is that taken by post-functional cracking.

Such cracks propagate by joining existing prism boundary discontinuities across the shortest interval and in a region where it is only required to separate crystals through the intervening matrix, rather than by breaking crystals. The fracture plane requires the minimum of creation of new surface. Only marginally more boundary needs to be exposed to create a 30° oblique fracture, and the extra part is of the head-on crystal-crystal orientation type (Boyde 1976, 1978b, 1989, 1990).

Breaks in the transverse direction through typical pattern 3 enamel must involve tensile (pull out) fracture of the interpit phase crystals (the 'tail' of the prism, if it can be described as 'tadpole shaped' in transverse section). Thus, breaks in this sense are hardest to generate and this aspect of interlocking of the tissue explains its relative

resistance to rapid prism boundary discontinuity exploitation loss, as, for example, in the sharp side of human incisors. When the prism boundary is steeply inclined to the developing enamel surface, several boundaries can be joined in transverse rows to form arcades (Fig. 1), and the transverse fracture may occur more easily.

Transverse fractures can also occur due to fatigue, and they may interconnect with both fractures exploiting the incremental planes and others following the 'easy' directions. In older individuals, these combinations lead to the loss of large blocks of enamel at the sharp edges of incisors and the shearing away of entire enamel walls in cheek teeth where dentine support has been lost due to cavity preparation or where there has been fatigue failure of the attachment of enamel to dentine.

The Hunter-Schreger bands are usually regarded as a further complexity of enamel structure that prevent the growth of an incipient crack. Great intricacy is seen in the teeth of carnivores, where there is a characteristic long-term enamel survival (low wear rate) and utilization of high local stress (e.g. in bone cracking) (Kawai 1955). In humans, the twisting complexity of the Hunter-Schreger bands is important for the survival of fillings placed in large cavities, where much of the enamel remains unsupported by enamel and/or dentine.

Cracking in enamel is cumulative: the numerical density and severity increase with use and, therefore, with age. Such cracks in humans are filled by the deposition of calcified bacterial dental plaque, material regarded as undesirable when only applied to external tooth surfaces. However, it is unlikely that this natural repair mechanism does much to repair the loss of any element of tensile strength within the tissue.

The extreme development of Hunter-Schreger bands in rodent incisor enamel is most likely a functional specialization to ensure a high rate of tissue loss, but in a pre-defined plane in order to maintain cutting edge sharpness. Whole transverse sheets of decussating prisms cleave away in parallel with the sheet boundary in the inner enamel. The extra incisal inclination of the outer enamel prisms (where Hunter-Schreger band-type decussation no longer occurs in rodent incisors) leads to an even sharper cutting edge of cleavage following the prism direction, or to a blunter, but stronger, bevelled edge if the cleavage parallels the interpit phase surface-normal crystals.

Acknowledgements

I thank many colleagues for their collaboration (especially S. J. Jones), the earlier financial support of the Medical Research Council, the Science and Engineering Research Council and The Wellcome Trust, and the current support of the Veterinary Advisory Committee of the Horserace Betting Levy Board.

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DISCUSSION

Diekwisch: Has anyone ever performed three-point bending analyses or finite element analysis to predict what happens when enamel breaks?

Boyde: Our own work is not based on predictions. I have performed many experiments in which I prepared specimens of enamel in particular orientations and, using a scanning electron microscope (SEM), actually looked at the behavioural properties as they break (Boyde 1978).

Robinson: As bovine enamel develops, the relative size of the prisms, which first appear to be the same width as the sheets, seem to change so that they are wider than the sheets. Is this observation 'real' or is it an artefact?

Boyde: It is a real observation.

Robinson: How does this process occur?

Boyde: There is a differential release of enamel matrix in the two locations: more is released as a shared product between two ameloblasts and less is liberated at the ends of each ameloblast. If one were to look at the transmission electron microscopy of this, one would see that this is reflected in the cellular architecture.

Mann: Presumably there is a small amount of organic material left in mature enamel. Where does it reside and does it have an effect on crack propagation?

Boyde: There is a small amount between the crystals and it is concentrated in the prism boundary discontinuities. It is not concentrated in those locations at the initiation of enamel formation, but it is concentrated there during the re-mobilization of the matrix during the maturation of the enamel.

It is possible that the protein-rich region at the prism boundary might function both as a crack stopper and a crack propagator. When large pieces of enamel break off, they always do so by exploiting these naturally existing breaks in the tissue. Thus, the prism boundary discontinuities appear to be planes of fundamental weakness in the tissue, but they are probably also important in allowing for a certain amount of 'give' in the tissue.

Fincham: You partly answered the question I wanted to raise, which concerned the nature and amounts of proteins at the prism boundaries. At the prism boundaries are we simply looking at what people have previously described as prism sheaths, which are simple discontinuities in overall structure between different orientations of crystals, or do they really represent areas that contain higher levels of protein? What is the evidence that higher amounts of protein are present?

Boyde: It is possible to observe an accumulation of the protein in demineralized tissue using transmission electron microscopy.

You used the term 'prism sheath', but I was careful to avoid the use of this term throughout my presentation. A study of the literature to determine the origin of this term reveals that it describes the structure which develops after acid etching, i.e. partial demineralization, of the enamel. However, such a structure is artefactual and results from a reprecipitation phenomenon to each side of the original prism boundary discontinuity, which is then incorporated in the middle of the artefactual structure (Boyde 1990).

Sharpe: You mentioned that different enamel arrangements in different species are adapted to different types of feeding. Are those patterns intrinsic to the ameloblasts of a particular species? In other words, is a horse ameloblast different from a dog ameloblast?

Boyde: They make different types of enamel.

Sharpe: But is anything else involved; for example, on crystal formation?

Boyde: This is not known, and your speculation would probably be as good as mine. The movement of ameloblasts that controls prism decussation can be traced through the ameloblast sheet to the junction with the stratum intermedium, but we do not know what controls this movement.

Veis: I would like to ask a question about the prisms themselves. You described how they are arranged, but within a prism itself how uniform are the sizes of the crystals

from the site of their initial formation to the ends, and are there changes in their orientation?

Boyde: As far as I can tell, they are the same throughout the prism. However, you have reminded me of another point, which is that in some of the literature today, authors point to structures in SEM images of etched enamel and, erroneously, call them crystals. They are not single enamel crystals, but groups of crystals.

Veis: As these crystallites grow are the proteins incorporated within the crystals themselves or do they remain as surface proteins on the small crystals?

Boyde: They remain on the surface of the crystals.

Slavkin: The elegant SEM images of various mammalian species have been used to identify topography patterns that characterize various mammalian species. For example, the forming enamel surfaces of rodents is remarkably different from that of lagomorphs. Greenberg et al (1983) showed that the epithelial genotype controls the topographical pattern of the forming enamel matrix. They employed SEM analysis to assay reciprocal homotypic as well as heterotypic epithelial-mesenchymal tissue recombinations between mouse and rabbit embryonic molar cap stage tooth organs. In these studies, the dental papilla mesenchyme did not influence the shape or orientation of the enamel matrix of the epithelia. Moreover, the epithelial genotype appeared to control the intracellular cytoskeletal architecture that accommodates the epithelial genotype-specific enamel prism formations.

Boyde: It is also worth noting that enamel is essentially made as a fossil, and it is the only tissue in which we can study the whole of mammalian evolution at the scale of tissue organization.

Rosenbloom: Are the surfaces of the ameloblasts orientated differently in the rabbit and in the mouse, and do they both have Tomes' processes?

Slavkin: Columnar cells have a different morphology in cross-section, and the Tomes' processes are also different in cross-section. They are clearly a reflection of the genotype.

Boyde: It would have been better to do those recombination experiments with a small carnivore, such as a ferret, and a small ungulate, such as a mini pig or a chevrotain. There is a strong size relationship in these different patterns: the typical pattern 3 ameloblast has twice the cross-sectional area of the typical pattern 2 ameloblast. In contrast, the rabbit and mouse ameloblasts are not that different in size.

Deutsch: Are the elongated crystals forming enamel of the same thickness? Secondly, is the final number of crystals in maturing and mature enamel equal (approximately) to the number established during the forming stage of enamel development or do additional crystals form later, e.g. during the maturation stage?

Boyde: I do not have any data which suggest that there is a difference in crystal size between the most internal and the most external enamel. The enamel crystals are long and can extend at least over many micrometres within a single prism. Growing crystals may cross at some parts of prism boundaries, but we may deduce that some new enamel crystals are formed later, because we also observe that many crystals end at prism boundaries.

Slavkin: It is difficult to visualize these processes in a dynamic sense. Would you entertain the possible explanation that there is an extracellular scaffold which serves as a template for the patterns of crystal growth and orientation?

Boyde: I have been careful to avoid consideration of the macromolecular, ultrastructural level. The scaffold theory cannot be excluded at that level of resolution. The most important feature of enamel matrix that distinguishes it from other mineralizing tissue matrices is that the most of the matrix is lost during mineralization so, if it is a scaffold, it is an obligingly weak scaffold that disappears when the job is done.

Simmer: I would like to make a comment about the proteins at the prism boundaries. Porcine ameloblastin (sheathlin) has recently been cloned and anti-peptide antibodies have been raised against it. Immunohistochemistry demonstrates that ameloblastin is randomly distributed in the enamel matrix near the Tomes' process but is concentrated in the prism sheath space in the deeper enamel layers (C. C. Hu, M. Fukae, T. Uchida et al, unpublished results 1996). Takashi Uchida believes that these proteins have a role in preventing inter-rod crystallites from invading the adjacent rod.

Boyde: You have emphasized what I have already said, i.e. that when the protein is first secreted it does not accumulate and that the accumulation is the first step in the maturation process.

Simmer: No, it accumulates prior to the maturation phase, during the secretory phase.

Boyde: But a large part of maturation occurs synchronously with secretion. For example, the longest-surviving enamels, such as human enamel and elephant enamel, are those in which most of the enamel mineralization process occurs within a short distance from the secretory front (Rosser et al 1967). One cannot make assumptions about human enamel mineralization based on what happens during rodent enamel mineralization: they are different processes.

Simmer: It is my understanding that the extension and maturation of enamel crystallites occur separately. During crystallite extension (the secretory phase) the crystallites are still thin, and the spaces between crystallites are sufficiently large to accommodate large amounts of protein. It is unlikely that ameloblastin is forced into the prism sheath space by being squeezed out by crystal maturation, rather it diffuses to this space and is retained by hydrophobic interactions with other proteins.

Boyde: Enamel crystals grow rapidly in thickness in human enamel. Thus, they are soon not as thin as they were in the first instance: some movement of the organic matrix allows this to occur.

Mann: It's difficult for me to understand what's going on. I have an image of a cell moving in a particular direction and the *c*-axis of a crystal oriented in that direction. How far away is the cell from the growing crystal tip? And is the cell moving with the crystal following behind, or does it move to a particular position before the crystal starts to follow? That is, do cell movement and mineralization occur simultaneously?

Boyde: Yes, they do move at the same time. This also relates to the question that Dan Deutsch asked, i.e. do new crystals form later? Mostly, once the process is under way,

new mineralization is represented by the growth of pre-existing crystals into the new space (*Lebensraum*) created by the formation of new matrix. The gap between the cell membrane and the nearest crystal is small and it represents a time difference of about 15 min. This is a much shorter interval than in the bone system, where there is an interval of a number of days between matrix formation and mineralization.

Mann: I envisage that the cell is reeling out the crystal in its wake, as though it were walking backwards and secreting an organized structure at the same time.

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Structure and function of secretory ameloblasts in enamel formation

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Abstract. Secretory ameloblasts have multiple functions including the synthesis and resorption of enamel matrix proteins and calcium transport during enamel formation. We have examined these functions by means of cytochemistry and immunocytochemistry. Enamel proteins, amelogenins and enamelines are localized in the biosynthetic pathways of ameloblasts and in the forming enamel. Sulfated glycoconjugates are present in secretory ameloblasts. The distal junctional complex of ameloblasts may act as a permeability barrier to enamel proteins, thereby confining the secreted proteins to the growing enamel front. Secretory ameloblasts contain lysosomal enzymes in the Golgi lysosome endoplasmic reticulum system and also exhibit absorptive capacity, which might be associated with an early decrease in extracellularly degraded enamel proteins. Active calcium transport through the ameloblasts towards the growing enamel is indicated by the demonstration of Ca-ATPase activity along the plasma membranes. A calcium-dependent modulator protein, calmodulin, is localized in ameloblasts, suggesting that early enamel mineralization is dependent upon calmodulin-regulated Ca-ATPase in ameloblasts. These results suggest that the secretory ameloblast is a highly specialized multifunctional cell in the production, resorption and degradation of enamel matrix and in the active calcium transport essential for matrix mineralization during enamel formation.

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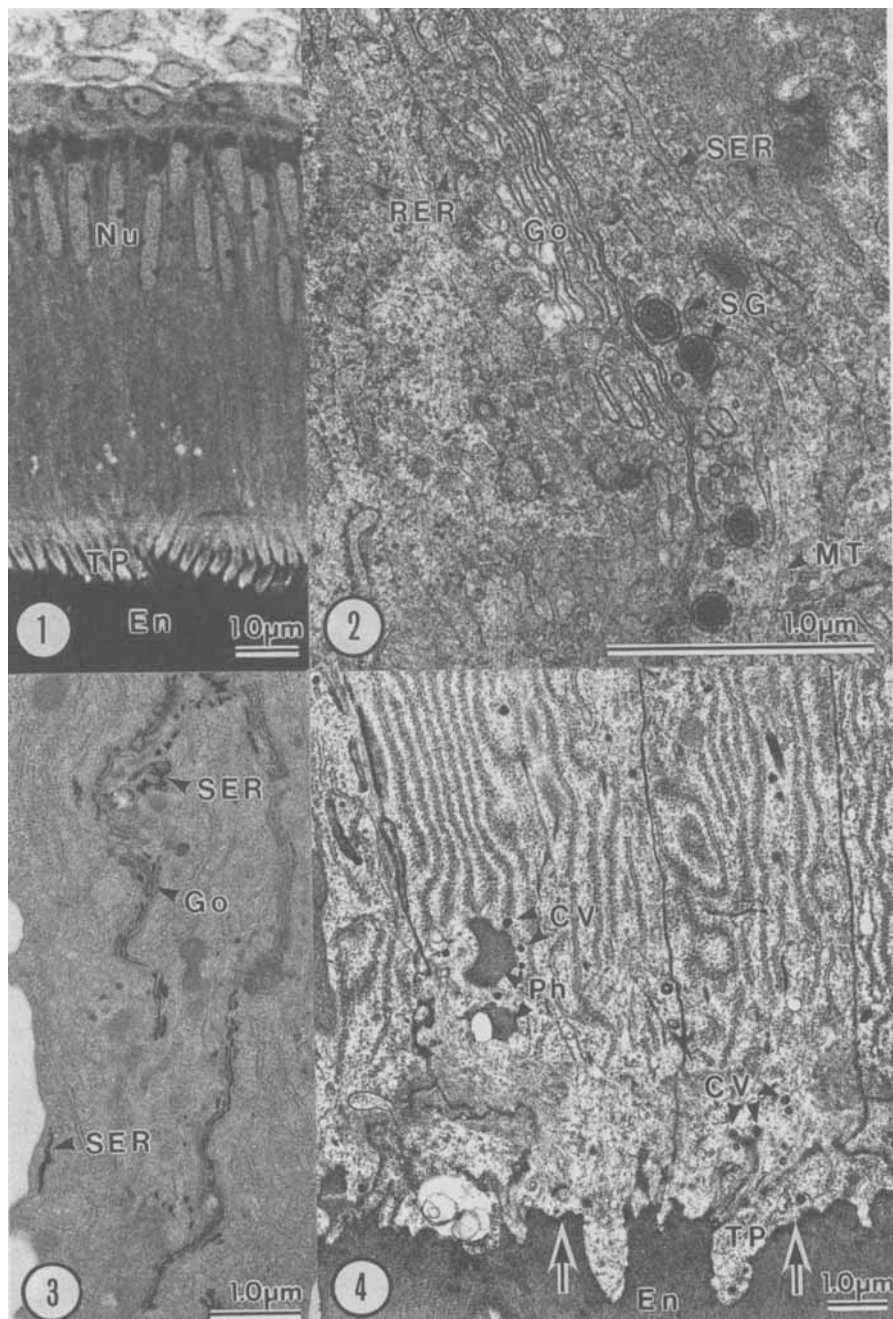
Mature enamel, which is the most highly mineralized tissue in the body, requires a long formation period from matrix production until the completion of maturation. The complex internal architecture of enamel is under the complete cellular control of ameloblasts, which are the cells that produce mineralized enamel matrix. Enamel matrix secretion begins shortly after the onset of dentine matrix mineralization. During enamel formation, secretory ameloblasts are directly involved in both the production and mineralization of enamel matrix (Boyde 1989, Sasaki et al 1990). Initially, secretion granules consist of enamel matrix glycoproteins — amelogenins and enamelines — which are responsible for the nucleation and regulation of enamel crystal growth (Nanci et al 1987, Aoba & Moreno 1989, Yanagisawa 1989, Deutsch

et al 1995). Although amelogenins are rapidly degraded and resorbed during the maturation stage (Nanci et al 1987), resorption of amelogenin breakdown products is thought to begin at the secretory stage via endocytosis from the Tomes' processes of secretory ameloblasts (Sasaki 1984, Nanci et al 1987). Transcellular calcium transport in early enamel mineralization is also regulated by a Ca-ATPase located in the plasma membranes of ameloblasts (Sasaki & Garant 1987, Sasaki et al 1987, Bawden 1989). To elucidate and visualize the cellular mechanisms of enamel formation, we describe the functional structure of the secretory ameloblast at the stage of enamel matrix secretion.

Fundamental structure of secretory ameloblasts

Secretory ameloblasts, which are tall and highly polarized cells, appear to be structurally suited for glycoprotein biosynthesis and secretion. In secretory ameloblasts many mitochondria are either concentrated in the narrow infranuclear cytoplasm, as in rodent tooth germs, or distributed throughout the cytoplasm, as in feline and human tooth germs (Warshawsky 1985, Sasaki et al 1990). The supranuclear cytoplasm contains a well-developed Golgi complex and extensive cisterns of rough endoplasmic reticulum (RER). The Golgi complex occupies a tube-like expanse of cytoplasm containing stacks of parallel cisterns, demarcating an inner core containing various types of vesicles, secretion granules, condensing vacuoles and elements of the smooth endoplasmic reticulum (SER) (Weinstock & Leblond 1971) (Figs 1.1–1.4). The membrane-limited secretion granules are present adjacent to the inner surface of the Golgi apparatus and at the distal end of the cell. Microtubules involved in the intracellular movement of secretion granules are concentrated longitudinally in the peripheral cytoplasm, and are also conspicuous adjacent to the Golgi saccules and within the Tomes' processes (Nishikawa & Kitamura 1985) (Fig. 1.2). The secretion granules fuse with the plasma membranes of the Tomes' processes, thereby releasing enamel matrix proteins into the extracellular spaces (Weinstock & Leblond 1971, Nanci et al 1987).

The secretory ameloblast has several zones of junctional specialization in its basal and lateral cell surfaces. At the proximal and distal ends of the cell bodies, ameloblasts are connected to one another by junctional complexes consisting of gap junctions, tight junctions and immature desmosomes. These junctions are essential for maintaining the organization of the ameloblast layer and in the control of metabolite diffusion along the extracellular spaces (Warshawsky 1978, Kallenbach 1980, Sasaki 1984) (Fig. 1.4). In freeze-fracture replicas, the proximal tight junctions are structurally leaky and incapable of forming a complete seal of the extracellular spaces. The distal zonular tight junctions exhibit a more extensive meshwork structure of junctional strands on the plasma membrane and can act as a permeability barrier to macromolecules such as enamel proteins, thereby confining the secreted proteins to the growing enamel front (Warshawsky 1978, Kallenbach 1980, Sasaki 1984) (Figs 1.4 & 2.5). The distal tight junctions may also prevent the movement of calcium ions through the extracellular spaces of ameloblasts (Bawden 1989).

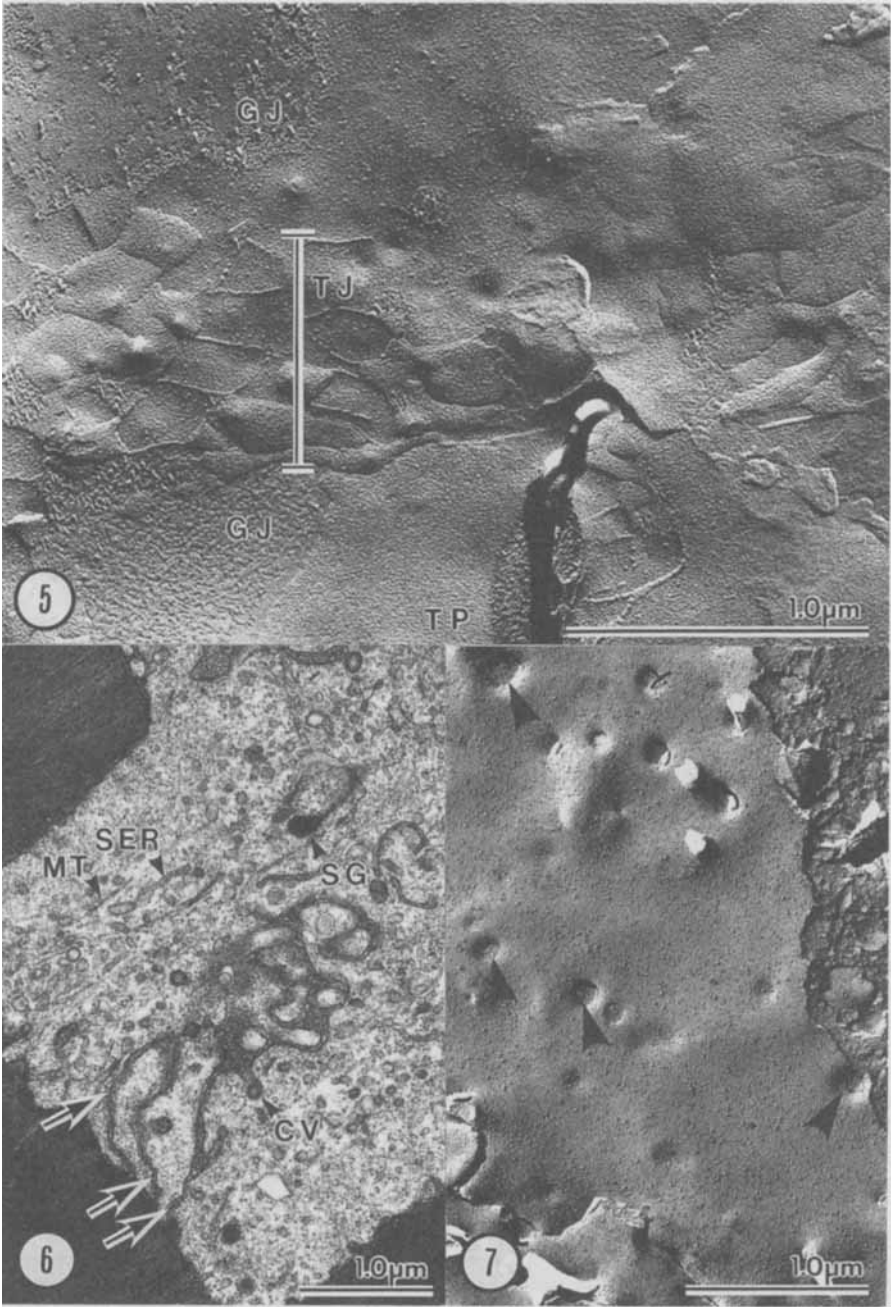


In the secretory ameloblast, upon the formation of the broad distal cell process (the Tomes' process), a dual area of secretion becomes operational. The Tomes' process contains many secretion granules, microtubules, microfilaments, SER cisterns, multivesicular bodies, lysosomes, coated vesicles and a few mitochondria (Sasaki et al 1990) (Fig. 2.6). The Tomes' process extends beyond the distal junctional complex and is the only cellular site to interact with the growing enamel surface. Specifically, the Tomes' process provides the sites of matrix secretion, matrix removal and mineral transport (Sasaki et al 1990) (Figs 2.6 & 2.7). Enamel secretion occurs at two sites in the Tomes' process: one is associated with the formation of rod enamel and the other with that of inter-rod enamel. Both secretory sites are characterized by deeply infolded plasma membranes, and are called rod and inter-rod growth regions (Leblond & Warshawsky 1979) (Fig. 2.6). The inter-rod growth region is formed by the proximal end of the Tomes' processes of contiguous co-operative ameloblasts. Here, enamel matrix accumulates between adjacent Tomes' processes to produce inter-rod enamel. The other site of enamel secretion corresponding to the rod growth region is located at the distal end of the Tomes' process of each individual ameloblast (Warshawsky 1985). At the border between the rod and inter-rod enamel, a sheath-like zone of slightly more concentrated organic matrix persists. This sheath corresponds to the non-secretory surfaces of the Tomes' process and demarcates the rod enamel from the inter-rod enamel.

Enamel matrix biosynthesis and secretion

Enamel consists of 20–30% protein in the early stage of amelogenesis, and the proportion of these proteins gradually decreases during the process of enamel mineralization. Enamelins are closely attached to the crystal surface and form envelopes around the individual crystals, whereas amelogenins are located in the intercrystalline spaces (Nanci et al 1987, Yanagisawa 1989) (Figs 3.8–3.10).

FIG. 1. (*opposite*) (1.1) Whole view of rat secretory ameloblasts. Oval nuclei (Nu) are located in the proximal portions of cell bodies and, from the distal ends, the Tomes' processes (TP) extend toward the forming enamel matrix (En) (from Sasaki et al [1990], courtesy of Karger). (1.2) The supranuclear cytoplasm of an ameloblast showing the Golgi (Go) apparatus, and the associated rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), cisterns and secretion granules (SG). Microtubules (MT) are also distributed in this area (from Sasaki et al [1990], courtesy of Karger). (1.3) Acid phosphatase (orthophosphoric monoester phosphohydrolase activated at acidic pH) activity, seen as electron-dense precipitates of lead phosphates in the Go and SER cisterns within the supranuclear cytoplasm of a secretory ameloblast (from Sasaki et al [1990], courtesy of Karger). (1.4) Injected peroxidase activity at the distal ends of ameloblasts in the early stage of enamel secretion. Electron-dense reaction products fill the narrow extracellular spaces and are also seen along the Tomes' process surfaces (arrows) facing the En. Some coated vesicles (CV) and phagosomes (Ph) appear to contain reaction products. Picture taken 20 min after peroxidase injection (from Sasaki et al [1990], courtesy of Karger).



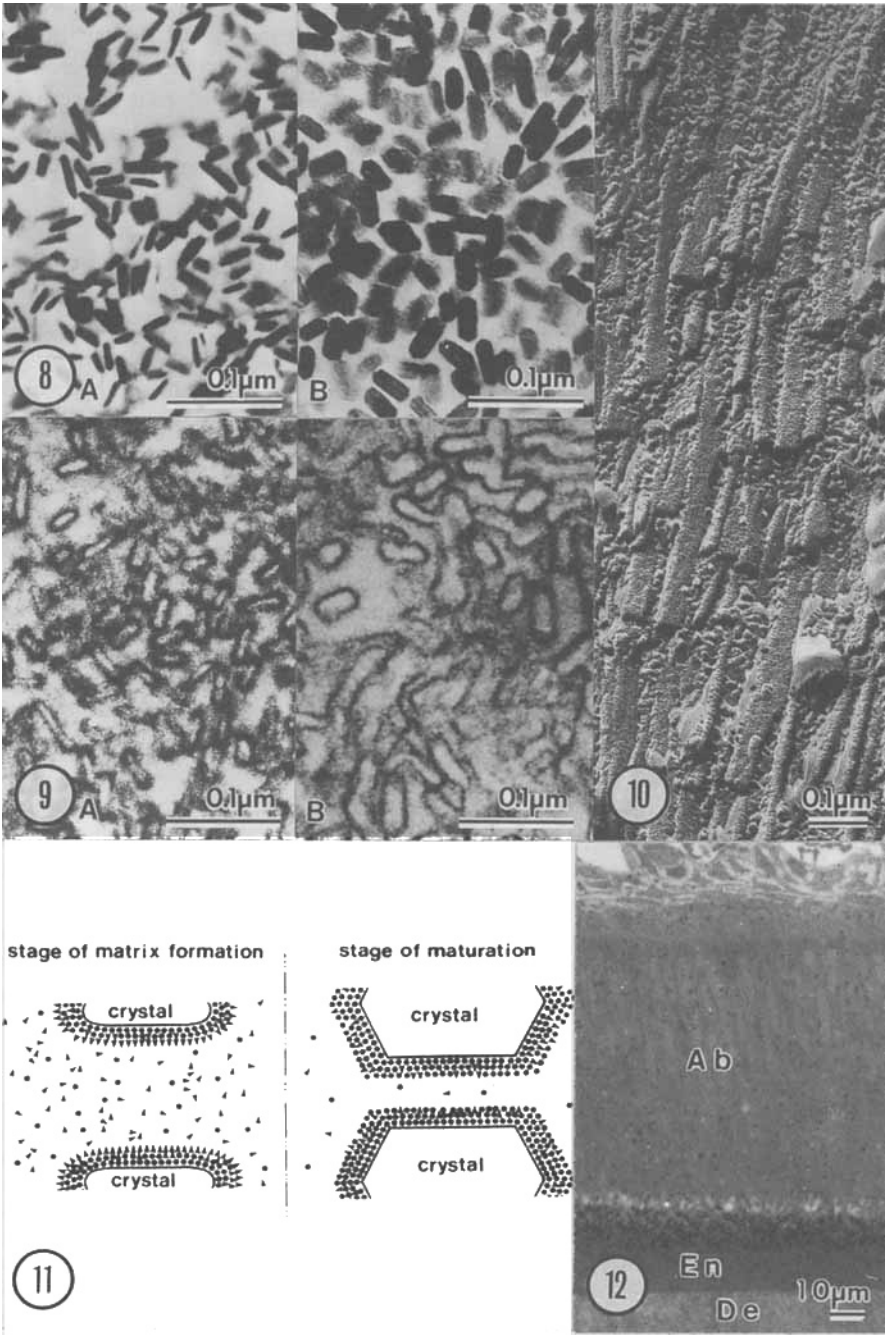
Ameloblasts synthesize and secrete these enamel proteins, particularly acidic enamelines, immediately before the appearance of apatite crystals. Such acidic enamelines have been hypothesized to have a role in the nucleation of enamel crystals (Deutsch et al 1995), whereas amelogenins are postulated to be involved in the control of crystal growth (Aoba & Moreno 1989) (Fig. 3.11). These enamel proteins are, therefore, regarded as participating in enamel mineralization through the promotion of nucleation and regulation of apatite crystal growth.

In studies of the main steps of enamel protein biosynthesis and the time dimension of the molecule transfer, light and electron microscopic autoradiographic studies with radioactive amino acids (Frank 1970, Slavkin et al 1976) and hexose sugars (Weinstock & Leblond 1971) have demonstrated that the protein portion of the enamel matrix is manufactured on the ribosomes of RER cisterns within 5 min and, shortly thereafter, transferred to the Golgi complex where, after glycosylation of the proteins, the enamel proteins are concentrated and packaged into secretion granules within 10 min. Utilizing both the amino acids and the carbohydrate components, the forming enamel is labelled within 30 min (Fig. 3.12). Although this autoradiographic technique has a non-specific limitation in that more than one cellular product is labelled, recent immunocytochemical studies have provided direct *in situ* localization and visualization of enamel proteins within specific intracellular and extracellular sites of secretory ameloblasts.

Neither an anti-amelogenin monoclonal antibody with a molecular weight of 27 kDa nor an anti-enamelin polyclonal antibody cross-reacted with its respective antigen in protein-A gold immunocytochemistry experiments (Shimokawa et al 1984). Specific immunolocalizations of both amelogenins and enamelines were detected as depositions of immunogold particles along the biosynthetic pathway of ameloblasts, i.e. in the ribosomes, RER cisterns, Golgi saccules, condensing vacuoles and secretion granules (Nanci et al 1987, Yanagisawa 1989) (Fig. 4.13). The forming enamel matrix surrounding the Tomes' processes was also intensely labelled by immunogold particles (Fig. 4.14). Such results are highly consistent with those of the previous autoradiographic studies described above.

The amino acids of enamel proteins are incorporated into peptides and larger proteins, which are further glycosylated and concentrated into specific secretion granules in the Golgi apparatus, and subsequently transported to the secretory pole

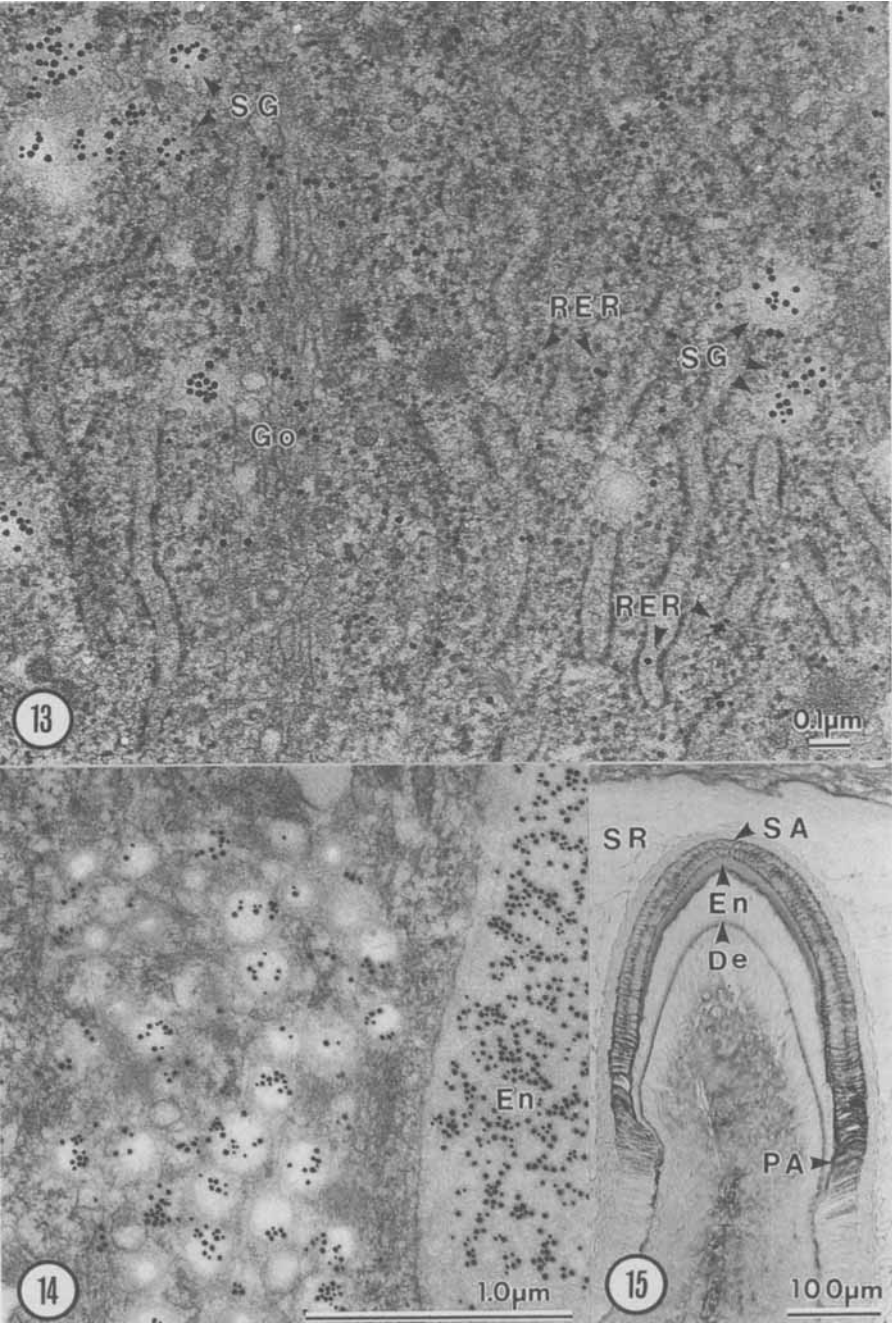
FIG. 2. (*opposite*) (2.5) Freeze-fracture replica of the distal junctional complex consisting of gap junctions (GJ) and tight junctions (TJ). Zonular tight junctional strands form an extensive meshwork structure proximal to the Tomes' processes (TP). (2.6) A distal portion of the rectangular Tomes' process of a feline ameloblast. Note the presence of deep membrane invaginations (arrows), in which the fusion of a secretion granule (SG) and a coated vesicle (CV) with the infolded membranes is seen. Many smooth endoplasmic reticulum (SER) cisterns and microtubules (MT) are also longitudinally distributed in the cytoplasm (from Sasaki et al [1990], courtesy of Karger). (2.7) Freeze-fracture replica of the Tomes' process. Round depressions with intramembranous particles, the sites of endocytosis (arrowheads), are seen on the plasma membrane P-face.



of the ameloblasts along a microtubular network (Kallenbach 1977, Nishikawa & Kitamura 1985). After synthesis, a portion of the enamel proteins is directly incorporated into multivesicular bodies and lysosomes, suggesting a down-regulation of secretory activity via post-translational degradation (Nanci et al 1989). Secretion occurs as the limiting membranes of the secretion granules fuse with specific areas of the plasma membranes in the Tomes' process. Although the secretion granules are believed to be released via exocytosis (Kallenbach 1977), some evidence indicates that the secretion granules may be released by direct fusion with the invaginations and tubular channels of cell surfaces at the secretory sites (Nanci & Warshawsky 1984) (Fig. 2.6).

Sulfated glycoconjugates other than enamelines and amelogenins are also localized in ameloblasts and the enamel matrix (Sasaki et al 1990). However, their precise cytochemical properties and biological roles in enamel formation are still unknown. Proteoglycans belong to a subgroup of the glycoconjugate category. The structures of proteoglycans are diverse with many core protein species and unique glycosylation patterns. There are, therefore, many different forms of proteoglycans such as versican, aggrecan, leucine-rich interstitial proteoglycans (decorin, biglycan, fibromodulin and lumican), perlecan, serglycin and syndecan. Syndecan-1 is involved in early tooth morphogenesis (Vainio et al 1991), but does not appear to be directly associated with enamel matrix formation. We recently identified versican localization in ameloblasts. Using a monoclonal antibody (MAb 5D5), which was derived from bovine sclera and specifically recognizes the core protein of versican (Bratt et al 1992, Larjava et al 1992), we observed strong immunostaining over the entire cell surfaces of pre-ameloblasts and secretory ameloblasts, as well as their intracellular granules, from human tooth germs at the bell stage. The cells of the stratum intermedium showed weak reactivity, whereas the cells of the inner and outer enamel epithelium and stellate reticulum were not immunostained (Fig. 4.15). Although the possible function(s) of versican in ameloblasts remains unknown, its precise subcellular localization is an interesting issue that awaits greater understanding of the roles of glycoconjugates in enamel formation.

FIG. 3. (*opposite*) (3.8) Cross-sections of oblong (A) and hexagonal (B) enamel crystals in the developing enamel of a rat incisor. These are undemineralized and unstained sections. (3.9) Cross-sectional components of enamel proteins in the developing enamel of a rat incisor. The compartment structures resemble the external contours of crystals, as shown in 3.8, and are altered in parallel with alternating crystal shapes. These components appear to form envelopes around each individual crystal. Sections were treated with phosphotungstic acid. (3.10) Freeze-fracture replica of enamel crystals and tightly bound granular enamel proteins. (3.11) Schematic illustration of possible sites of amelogenins (solid circles) and enamelines (solid triangles) at the stages of matrix formation and maturation in developing enamel. (3.12) Light microscopic autoradiograph of secretory ameloblasts (Ab) at 4 h after ^3H -proline injection. Silver grains are heavily accumulated in the newly formed enamel matrix (En), but small grains can be seen over ameloblasts. De, dentine.



Resorptive function

In developing enamel a gradual loss of enamel proteins together with an increase in smaller molecular weight materials occurs during the secretory stage (Robinson et al 1982). The enamel proteinases, which degrade amelogenins, have also been indicated to be either secreted or derived from latent proteinases that had been deposited in the enamel during the secretory stage (Smith et al 1989). Degradation of amelogenins is thought to be directly related to the progressive crystal growth in forming enamel (Aoba & Moreno 1989). Partially degraded enamel proteins have been suggested to undergo further resorption by secretory ameloblasts (Smith 1979, Kallenbach 1980, Sasaki 1984, Nanci et al 1987).

As a demonstration of endocytotic activity, when horseradish peroxidase was administered as a soluble protein marker, it rapidly penetrated up to the ameloblast-enamel matrix border only at the initial stage of matrix formation and was observed in coated pits and vesicles in the Tomes' process (Fig. 1.4). Freeze-fracture replication of the Tomes' processes revealed the presence of many round depressions in the plasma membrane P-face with accumulations of intramembranous particles, suggesting an endocytotic site (Fig. 2.7). The injected peroxidase is subsequently incorporated into pale endocytotic vacuoles and larger phagolysosomes (Fig. 1.4). The endocytotic pathway demonstrated with peroxidase in ameloblasts may parallel the resorptive process of enamel proteins and/or their degraded fragments (Sasaki 1984).

In this regard, Nanci et al (1987, 1989) demonstrated the presence of enamel proteins in lysosomes and multivesicular bodies of secretory ameloblasts. It is, however, still uncertain whether such enamel proteins in lysosomes are of intracellular or extracellular origin. If the immunoreaction in lysosomes of secretory ameloblasts represents resorption of enamel proteins from the forming enamel matrix, resorbed proteins can be considered to be further degraded and digested within lysosomes. In fact, the enzymatic reaction of acid phosphatase as a lysosomal marker is detected in the Golgi apparatus, Golgi-related SER cisterns and lysosomes in the supranuclear cytoplasm and the Tomes' process of ameloblasts (Fig. 1.3). The absorptive and digestive activities of secretory ameloblasts are considered to be associated with an

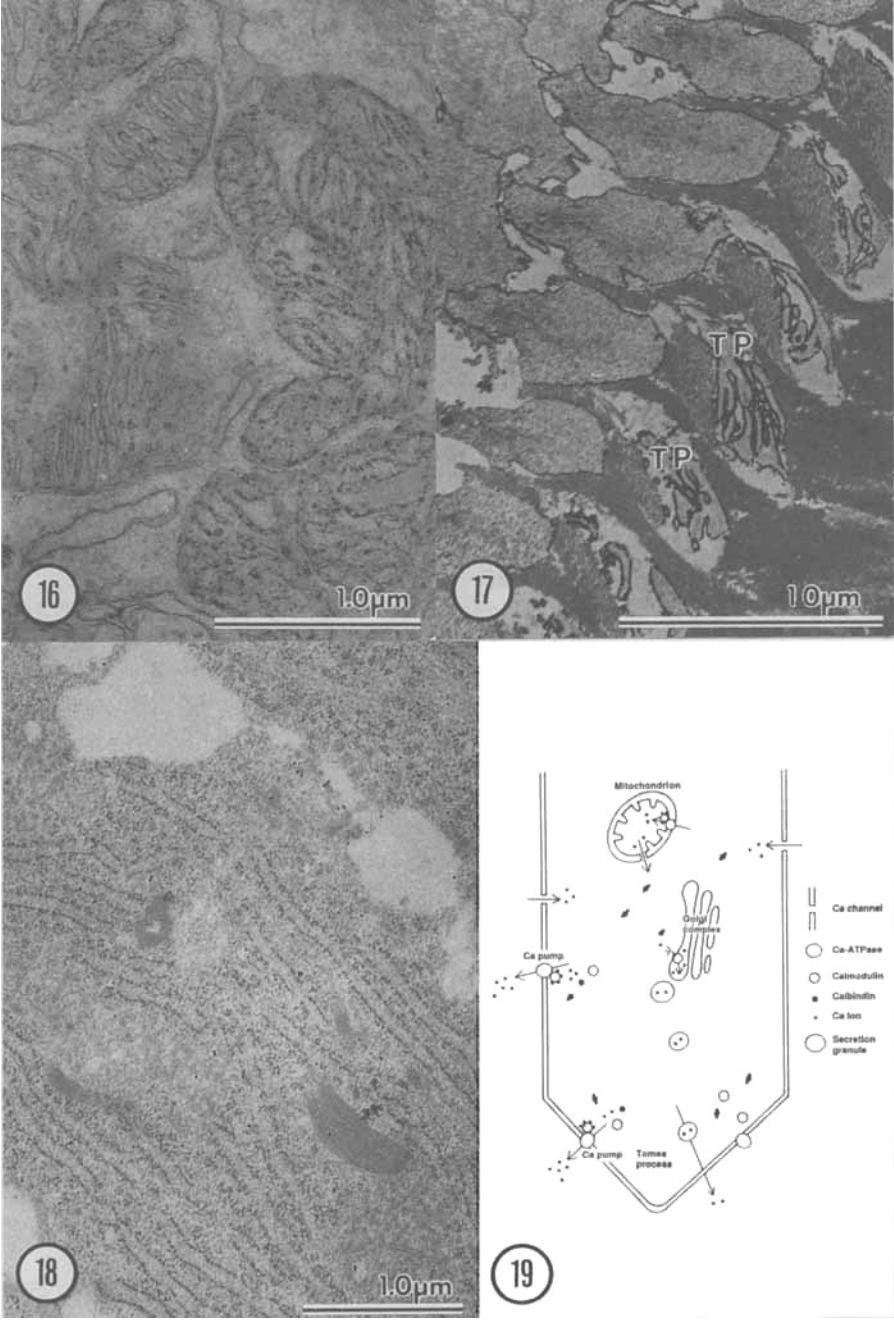
FIG. 4. (*opposite*) (4.13) Amelogenins and enamelines in the Golgi area of an ameloblast. Large immunogold particles, which reacted with amelogenins, are observed in the rough endoplasmic reticulum (RER) cisterns, Golgi (Go) cisterns and secretion granules (SG). Small immunogold particles that have reacted with enamelines are seen in the same cytoplasmic organelles. (4.14) Enamel protein localization in the Tomes' process and enamel matrix (En). A single secretion granule contains both large (amelogenin-related) and small (enamelin-related) immunogold particles. Numerous large and small immunogold particles are located in the enamel matrix. (4.15) Light micrograph of a 6 μ m section of a human tooth germ at the bell stage. Immunoperoxidase staining with the monoclonal antibody MAb 5D5 to versican. Strong staining is visible in the pre-ameloblasts (PA) and secretory ameloblasts (SA), but most cells of the inner and outer enamel epithelium, stellate reticulum (SR) and stratum intermedium are not immunostained. De, dentine.

early decrease in proteins and/or their degraded fragments in the enamel matrix, which is necessary for further enamel mineralization.

Calcium transport

Calcium localization and movement through secretory ameloblasts towards the forming enamel has been examined using ^{45}Ca autoradiography and calcium precipitation techniques (Nagai & Frank 1975, Leblond & Warshawsky 1979, Eisenmann et al 1982, Reith & Boyde 1985, Kogaya & Furuhashi 1986). Concerning the extracellular and transcellular routes of calcium transport, diffusion of calcium ions through the extracellular spaces of secretory ameloblasts may be prevented by their distal tight junctions (Warshawsky 1978, Sasaki 1984). Active transcellular calcium transport within ameloblasts towards the enamel is a concept supported mainly by cytochemical and immunocytochemical studies (Sasaki & Garant 1987, Sasaki et al 1987, Borke et al 1995, Takano 1995). The ATP-dependent calcium pump, Ca-ATPase, has a high affinity for calcium and is involved in the active efflux of calcium ions out of cells against a concentration gradient, thereby creating a high local concentration of calcium ions needed to induce precipitation of calcium phosphate salts in the vicinity of ameloblasts. The enzymatic activity of Ca-ATPase has been demonstrated in the mitochondria, Golgi saccules and plasma membranes of ameloblasts (Sasaki & Garant 1987, Sasaki et al 1987) (Figs 5.16 & 5.17). The site of enzymatic activity is highly consistent with the calcium localization in ameloblasts (Nagai & Frank 1975, Eisenmann et al 1982, Reith & Boyde 1985, Kogaya & Furuhashi 1986). Ca-ATPase activity along the mitochondrial cristae is also consistent with the known accumulation of calcium ions by these membranes in the inner compartments via ATP hydrolysis, which regulates the cytoplasmic calcium ion concentration. Ca-ATPase in the Golgi saccules may condense calcium ions into secretion granules, thus functioning in the nucleation of mineral crystals in newly formed enamel (Eisenmann et al 1982). However, the continuous calcium supply for crystal growth is thought to be provided by an active transport mechanism (Sasaki et al 1987, Bawden 1989, Takano 1995). The most intense Ca-ATPase activity and its gene expression are localized along the plasma membranes of the lateral and distal cell surfaces, and particularly along the infolded membranes of the Tomes' processes (Sasaki & Garant 1987, Sasaki et al 1987, Borke et al 1995) (Fig. 5.17). These results point to the presence of an active calcium pump in the Tomes' process, where a continuous calcium supply is required for the normal progression of apatite crystal growth.

FIG. 5. (*opposite*) (5.16) Ca-ATPase activity along the mitochondrial cristae (from Sasaki et al [1990], courtesy of Karger). (5.17) Ca-ATPase activity along the cell surfaces of Tomes' processes (TP) (from Sasaki et al [1990], courtesy of Karger). (5.18) Calmodulin localization in secretory ameloblasts. Immunogold labelling is observed on polyribosomes and the cytoplasmic matrix close to the plasma membranes. (5.19) A proposed model for calcium transport in secretory ameloblasts (from Sasaki et al [1990], courtesy of Karger).



The calcium ion concentration in the cytoplasm is estimated to be between $10^{-6.5}$ M and $10^{-7.5}$ M, whereas the concentrations in the extracellular fluid adjacent to the proximal cell surfaces and the enamel fluid adjacent to the distal cell surfaces of secretory ameloblasts are 10^{-3} M and 10^{-5} M, respectively. The differences in calcium ion concentration among these compartments may explain the net influx of calcium ions at the proximal portion of ameloblasts and the net efflux of calcium ions at the distal portion, the Tomes' process (Bawden 1989).

Secretory ameloblasts also contain various intracellular calcium-binding proteins (CaBPs), such as calbindin-D 28 kDa, calbindin-D 9 kDa, calmodulin, annexins and parvalbumin (Sasaki et al 1990, Berdal et al 1991). Although the precise role of CaBPs in ameloblasts is not yet known, calcium ions in the surrounding tissue fluids would presumably enter the cytoplasm of ameloblasts through the proximal and lateral plasma membranes along a concentration gradient and bind to CaBPs with high affinity as a buffering system (Sasaki et al 1987, Berdal et al 1991). In this regard, Ca-ATPase expression is not found in the proximal portions of secretory ameloblasts (Borke et al 1995). The calcium-CaBP complexes would then move towards the distal cytoplasm and, after release from CaBPs, calcium ions may be extruded towards the enamel matrix by the Ca-ATPase in the Tomes' process membranes. However, based on its localization, calbindin-D 28 kDa has been postulated to be involved in the regulation of intracellular calcium homeostasis, signalling and cytoskeletal maintenance rather than the active transport system (Berdal et al 1991). On the other hand, the calcium-dependent modulator protein calmodulin is thought to be an activator of membrane-bound Ca-ATPase through the formation of a calcium-enzyme-calmodulin complex. Calmodulin distribution has been demonstrated in polyribosomes, the cytoplasmic matrix and along the plasma membranes of ameloblasts (Sasaki et al 1987, Sasaki et al 1990) (Fig. 5.18). It is thus postulated that secretory ameloblasts possess a calmodulin-regulated calcium extrusion pump and are involved in transcellular calcium transport for enamel mineralization, as well as regulating the cytoplasmic calcium ion concentration (Fig. 5.19).

Summary

The ameloblast is a highly polarized protein secretory cell that compartmentalizes the extracellular space, creating a microcompartment for matrix polymerization and mineralization. It also participates in the removal of breakdown products of the enamel matrix and appears to transport calcium actively to the mineralization front.

Acknowledgement

We wish to thank P. R. Garant, School of Dental Medicine, State University of New York at Stony Brook, for his valuable advice.

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DISCUSSION

Wright: How do cells maintain the junctions between two plates moving in opposite directions which are necessary to regulate ion concentrations and influx of other materials that might inhibit crystal growth?

Sasaki: This is not known. Warshawsky (1978) demonstrated that the cells have two types of junctional complex: either at the proximal end or the distal end of cell bodies. The tight junctions are more extensive in the cells between the rows of ameloblasts and less extensive in the cells between the cells within a row.

Wright: Do these junctions have a role in cell movement?

Sasaki: I don't know.

Robinson: I would like to make a comment about whether particular proteins bind to apatite. There are two ways of determining this: (1) by removing the soluble protein and looking at the remaining protein; or (2) by exposing 'naked' enamel crystals to mixtures of enamel matrix proteins. In the first case, however, one may have removed a protecting protein (possibly amelogenin) from the crystal surfaces and allowed another protein to bind, which doesn't normally have access to crystal

surfaces. It's dangerous, therefore, to draw the conclusion from this experiment that a particular protein normally binds to apatite *in vivo*. The second approach doesn't tell you anything about the situation *in vivo* because the preparation of soluble matrix proteins may permit access of proteins to crystals that are normally not present at crystal surfaces. This is a difficult area and we often talk about these experiments as if we've identified specific crystal-bound proteins, which I don't think we necessarily have.

Fejerskov: Can you speculate on what controls the formation of the Tomes' processes? When the Tomes' processes are knocked out experimentally, the enamel crystals have a parallel carpet-like arrangement.

Sasaki: The control mechanism behind the formation of the Tomes' processes is not known. However, we have some evidence concerning the role of Tomes' processes, which are responsible for the formation of rod and inter-rod enamel. In mice susceptible to osteosclerosis (*oc/oc*) the bone marrow is not formed because the osteoclasts do not have ruffled borders for mineral dissolution. Similarly, the ameloblastic Tomes' processes do not have membrane infoldings at the distal end. I originally thought that one function of the enamel matrix was to promote the formation of the Tomes' processes, but this is not the case because the Tomes' processes are formed in the absence of membrane infoldings.

Rosenbloom: Is the genotype of this strain of mouse known? Because *sre*-deficient mice have the same phenotype, i.e. teeth do not erupt on the surface of *sre*-deficient mice. It is possible that they do form normally, but just don't come to the surface.

Sasaki: The *oc/oc* mouse has an osteosclerotic phenotype but the gene responsible is not known.

Fincham: The assertion was made in your presentation that the crystals were encased in a protein sheath. I would like to challenge that concept by asking everyone to think about what the surface area of crystal might be in the early stages of enamel development, and what amount of a protein might be required to make a monomolecular coat on that surface. In my opinion, it is highly unlikely that the crystals are encased in protein because too much protein would be required.

Sasaki: But when we can't even see tiny crystals, we still see a certain amount of enamel proteins in the matrix. This led us to suggest that the production of enamel proteins preceded the precipitation of calcium phosphate salts.

Deutsch: It is not necessary for the entire crystal to be covered with proteins. Accumulation of a specific protein (e.g. one that could act as a template) at the site of initial mineralization (see, for example, Traub et al 1985) might be important for providing a favourable environment in which the initial crystal is formed. The proteins might not remain for any other reason than to promote and regulate the initial stages of mineralization. They may then degrade to enable the crystal to grow in width and thickness.

In this sense it is interesting that Falini et al (1996) reported that macromolecules extracted from the aragonitic shell layers of some mollusc shells induced aragonite formation *in vitro* in a controlled microenvironment, whereas macromolecules of calcitic shell layer induced calcite formation under the same conditions.

Robinson: The statement that much of the matrix function is simply to nucleate crystals is an oversimplification. It is concerned with the architecture of the tissue—for example, the size, shape and disposition of the crystals—and not just how the crystals may be initiated. Apatite is one of the most stable calcium phosphates, so then one could ask the question how much encouragement does it need to form? In addition, the chemistry of enamel apatite is extremely variable: the outermost enamel has about 1% carbonate, whereas it increases to 4% in the interior (and there are also variations in the concentration of magnesium ions). Therefore, the same apatite is not being produced throughout the entire process of enamel development.

Mann: Why does calcium uptake occur on the proximal side of the cell and calcium efflux on the distal surface? Is an antiport system present, so that protons released by the mineralization process are transported against calcium through the channel?

Sasaki: The enamel organ cells have various kinds of ATPase activities. For example, sodium pumps are present on the plasma membranes of the cell surface of ameloblasts, so some kind of exchange system such as a $\text{Na}^+ - \text{Ca}^{2+}$ exchange may also be operating.

Mann: It would be interesting if there was some sort of feedback mechanism for mineralization involving calcium ions.

Diekwisch: I have two questions. First, I have been interested in what happens to the water in the developing enamel matrix. Recently, people have speculated that some of the small proteoglycans may bind to this water, and I wondered whether you have done a time-course study to determine whether these proteoglycans are up-regulated and then down-regulated?

Sasaki: We only have data for the secretion stage and have not looked at the maturation stage. During the maturation stage, the water is removed as a consequence of sodium transport and the Na-K-ATPase proteins are apparently localized around the cell surface of maturation ameloblasts. This phenomenon may involve calcium ion uptake by the matrix.

Diekwisch: My second question relates to the pattern of immunogold distribution in the enamel layer. The gold dots are relatively small compared to the large IgGs that are used as secondary antibodies, but there are still specific patterns of gold dots throughout the enamel layer. What does this mean?

Sasaki: We have not yet repeated this experiment using secondary antibodies. We have compared the results of the protein A complex with a secondary antibody conjugated with immunogold particles, and there are sometimes differences between these results.

Butler: I have a point concerning the proteoglycan versican, which you showed was secreted by pre-ameloblasts and ameloblasts but not by cells earlier in the same lineage. As you know, versican is found in many different tissues and is not unique to ameloblasts. This situation of mixing and matching of proteins occurs in a number of tissues. For example, although there are two or three proteins considered unique to bone, the majority of bone proteins are expressed in numerous other tissues as well. Enamel is fairly unusual in that it has several unique proteins not found in

other tissues, but the same mixing and matching process seems to occur since ameloblasts secrete dentine sialoprotein, dentine matrix protein 1 and versican. This idea suggests that in individual tissues there are complex regulatory systems operating that select a precise mix of proteins necessary for forming the tissue, rather than the individual proteins *per se*.

Snead: I would like to comment on your observations that enamelin and amelogenin co-localize within a single secretory vesicle. Using the two-hybrid system, we have not been able to demonstrate an interaction between amelogenin and tuftelin, so I was surprised that the vectorial transport of enamelin and amelogenin occurs within the same secretory vesicle. Have you considered what that means in terms of the organization of the forming front of the extracellular matrix (ECM)? The enamel ECM seems to have demands for compositional differences, i.e. there are territorial domains within the ECM that should have different stoichiometries. Is this stoichiometry achieved by the selection of various amelogenin isoforms, combined with the tuftelins and other enamel proteins?

Sasaki: There are both amelogenins and enamelins in the secretory granules, but this does not necessarily imply that there is an interaction between amelogenins and enamelins in an individual secretory granule. However, we have not identified secretory granules that contain either amelogenin or enamelin.

Zeichner-David: Pre-ameloblasts do not synthesize enamel proteins. Pre-ameloblasts (which are still dividing and are not fully differentiated) first synthesize tuftelin, followed by ameloblastin and then amelogenin. This occurs prior to the secretion of enamel proteins. I also would like to know which antibodies you used for your enamelin and amelogenin localization studies, because what we used to call enamelins are now tuftelins, ameloblastin and various proteases.

Sasaki: H. Shimokawa made the antibodies. They were raised against 27 kDa amelogenins or 50–70 kDa enamelins.

Deutsch: We have recently published a paper in which we described the results of double-labelling experiments using a monoclonal antibody raised against amelogenin and a polyclonal antibody raised against tuftelin (Deutsch et al 1995). We found that they do not co-localize in the cell. Also, in our experience, these antibodies sometimes cross-react weakly with the stratum intermedium. Does anybody know why this occurs?

Nanci: In our experience, when we see antibody cross-reactivity in the stratum intermedium it's usually associated with patches of material located among the interdigitations of the stratum intermedium cells. We have not yet seen labelling inside the Golgi apparatus or inside secretory granules of these cells. Some enamel proteins are also found between ameloblasts and stratum intermedium cells. Since secretory granules containing enamel proteins are occasionally present in the infranuclear compartment, it is conceivable that they may release their contents basally among stratum intermedium cells (Nanci et al 1985, 1987, Nanci & Smith 1992).

Snead: I would just like to comment that when doing *in situ* hybridization analyses under stringent conditions, we occasionally saw stratum intermedium cells that were

labelled. It seems as though there is a short burst of activity, but I'm not sure what it means because it is so transitory.

Diekwisch: Perhaps it is a problem associated with the preparation of the samples because at that point in time ameloblasts are heavily loaded with amelogenins, so that any damage during the preparation may result in some of the proteins being translocated into the area.

Nanci: But we haven't observed any evidence of damage when enamel proteins are found basolaterally. Secretory granules are normally found in the proximal part of secretory stage ameloblasts and these likely account for the basal release of enamel proteins.

Zeichner-David: I have a comment relating to what we have been calling the differentiation between the secretory ameloblast and the maturation ameloblast. In my opinion the differences are extremely complicated. C. E. Smith and A. Nanci have demonstrated numerous times that ameloblasts in the maturation state are still secreting proteins. Therefore, it is a dynamic process in that the cells are secreting and resorbing at the same time. How can you differentiate between secretion and maturation with the types of experiments that you are doing?

Veis: This is really a semantic argument because maturation is a progressive event. Even a cell that is just beginning to become secretory is doing more than just that. As a cell becomes older, it may synthesize a different set of proteins, but it also may continue to make proteins characteristic of the less mature stage.

Butler: Also, when considering the role of proteases in the formation of enamel, one must remember that proteases are not usually secreted in an active form. Rather, they are initially secreted in an inactive form or an inhibited form and are activated at the position or time where needed.

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General discussion I

Aldred: I would like to raise a point about the terminology we currently use for identifying ameloblast stages. At the moment we are using terms because they are easy to apply but the actual situation is not that simple. In my opinion we should stop using this prejudicial terminology.

Slavkin: Also, we're not talking about a homogeneous monolayer of ameloblasts. The assumption is that there are populations of cells within the inner enamel epithelium which are moving in three dimensions during the process of enamel production. During these transitions, cells function as either secretory, or 'clastic' groups, or clusters of cells. Does anyone have any direct evidence to show a cell moving within the inner enamel epithelium?

Nanci: In the rat incisor Smith & Warshawsky (1975) have demonstrated using ^3H -thymidine labelling that ameloblasts are carried passively with the erupting tooth from the apical end towards the incisal end.

Zeichner-David: Are the cells moving by themselves or are they being displaced? Because these are quite different processes.

Nanci: There are two different motions: an apical-incisal passive movement and the lateral sliding of cells from adjacent rows (Warshawsky 1978). In the 'sliding row hypothesis' a puzzling point is what happens to ameloblasts as they slide past each other and reach the lateral extremity of the incisor.

Slavkin: Alan Boyde suggested that there are populations of cells working in aggregates or units which are integrated into the enamel formation process (Boyde 1997, this volume). Individually, these units have either a different direction or a different rate of activity.

Boyde: There are as many different enamels as there are mammals, so we should be careful about making generalizations. In forming rhinoceros inner enamel there are two populations of cells, one of which is moving down the tooth and is trying to join an embryologically younger population, and the other is moving up the tooth to join an older population. At the end of the secretory phase these two different populations enter the maturation stage at correspondingly different time intervals (Boyde & Fortelius 1986).

Slavkin: Irma Thesleff presented evidence that pre-ameloblasts and ameloblasts express bone morphogenetic protein (BMP) 5 and BMP2. If we take into consideration the evidence that Alan Boyde has just presented, and if we carefully examine the immunolocalization patterns, we may discover that there are different growth factors and that their cognate receptors are distributed in clusters of cells,

rather than having a homogeneous distribution in all pre-ameloblasts. Therefore, it is possible that terminologies such as pre-ameloblast or ameloblast, or secretory versus resorptive, may not serve to best describe what is being discussed. We need to integrate biology.

Deusch: On the transition from the forming to maturing stage, the ameloblasts begin a series of rapid modulation cycles between two types of cell morphologies (ruffle-ended and smooth-ended) along the surface of maturing enamel. Do these represent two different types of ameloblast cells that receive different signals at the forming stage, or is the same cell changing its morphology and activity in a cyclic manner?

Veis: If one considers tooth formation, starting off at the cap stage, the cells at the most coronal aspect are already differentiated and more mature. As the cells undergo duplication and extend apically as the rest of the crown structure is built, there may be a change in signalling that defines the shape of the tooth. The ameloblasts may change their phenotype according to their position along the crown. In human teeth the incidence of caries is often higher on one side of the tooth than the other, so there must be architectural phenotypic differences for every tooth. It may not necessarily be true that all the ameloblasts follow exactly the same programme at all times because they start off at different stages and in different environments.

Aldred: I'm a little concerned about the way this discussion is going. We are hearing suggestions that there are different populations of cells, but why cannot we just say that ameloblasts are smart cells and do the job that they're intended to do rather than trying to classify them into two different types.

Nanci: The ameloblast is indeed a smart cell. We have recently obtained evidence that some of the proteins they make are multifunctional, depending on the time at which they are released extracellularly (Sawada & Nanci 1995). They also make extracellular matrix proteins typically made by mesenchymal cells, which produce the extracellular matrix of collagen-based mineralized tissues. Indeed, this is what occurs at the enamel-free area of rodent molars, most likely during early cementum formation (Bosshardt & Nanci 1996).

Snead: The way that ameloblasts are organized on certain decussation lines appears to improve the masticatory function of herbivore teeth, for example. We know that there are many developmental signals that specify tooth formation. If there is pattern information in the jaws specifying the size and shape of the tooth, is there also pattern information among the ameloblasts that identifies another order of biological complexity and speaks to the different signals that orchestrate decussation angle and emergence rates, for example? If there is another level of cohorts of cells then there must be another level of organization. This level of organization must either involve an intrinsic pathway, which describes pattern formation at that level, or extrinsic epigenetic signals. A further level of complexity if these suppositions are correct is that downstream there must be yet another series of genes, which have undergone regulatory pressure to emerge across evolutionary time in these different types of orchestrated enamel prism formations.

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Structure, crystal chemistry and density of enamel apatites

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Abstract. The apatitic calcium phosphate crystals in dental enamel are too small for single crystal diffraction studies so the only possible direct structure determination must use whole-pattern-fitting Rietveld analysis of X-ray and neutron powder diffraction patterns. As a result, aspects of the structure are not known in detail. Further structural information can be obtained by consideration of published chemical analyses and infrared studies, taking into account studies of the crystal chemistry of synthetic apatitic analogues of enamel apatite. The apatitic constitutional water and total water content of enamel are particularly important, but there are difficulties in their determination. Making reasonable assumptions, a number of models of the unit cell can be derived. The weight per cent (including constitutional water) and density of the enamel apatite crystals for the most probable model are about 98 wt. % and 3.0 g cm^{-3} , respectively. The apatite volume per cent calculated from these values is about 96%. The weight per cent and volume per cent of enamel apatite are higher than normally accepted values because of inclusion of constitutional water and use of a density for enamel apatite that takes into account its known lattice expansion over hydroxyapatite and probable lattice vacancies.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 54–72

The structures of well-defined inorganic solids available as single crystals can easily be determined by standard diffraction methods. Unfortunately, the mineral in dental enamel is neither chemically well defined nor available as single crystals, so its structure can only be determined approximately by diffraction from powders and otherwise inferred from spectroscopic studies and analogies with model synthetics. The extensive literature on this subject will not be reviewed or cited in detail; for this the reader is referred to discussions by Simpson (1972), McConnell (1973), LeGeros (1991), Driessens & Verbeeck (1990) and Elliott (1994). In this chapter emphasis will be on information that can be derived from chemical analyses and analogies with model compounds. It will be assumed that dental enamel can be regarded as a two-phase system, the major one being an apatitic calcium phosphate and the other organic, principally proteinaceous, with additional H_2O . Thus, the possibility of there being more than one apatitic phase (Wöltgens et al 1980) or the presence of up to 10 wt. % of a magnesium whitlockite (Driessens & Verbeeck 1982) will not be discussed. In

addition, variations in composition due to changes in the surface composition of the crystals of the inorganic phase will be ignored: these possibilities are most likely to apply to the carbonate (CO_3^{2-}) and hydrogen phosphate (HPO_4^{2-}) ions.

A brief discussion of the structures of various model compounds is included. Unfortunately, single crystals are available for only a few, such as hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and fluorapatite, $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$. The most relevant model compounds for enamel apatite, the non-stoichiometric precipitated apatitic calcium phosphates and the carbonate-containing apatites occur only as powders (except for some mineral carbonate-containing apatites), so single crystal structural information is not available. Nevertheless, studies of the chemistry of these systems give much information about possible ionic substitutions.

For many elements, a discussion of the chemical analysis of enamel is straightforward and the results, in structural terms, can be interpreted without ambiguity. However, there is difficulty in determining the total H_2O content, its division between the apatitic and organic phases, and the extent to which the apatitic H_2O is ionized to give structural OH^- and H^+ ions (the latter forming HPO_4^{2-} and possibly HCO_3^- ions). One result of these difficulties is that there is uncertainty about the mass fraction of the inorganic phase and hence its volume fraction.

Also included is an assignment of the chemical species in a published analysis of enamel to the various sites in the apatitic unit cell in a way consistent with the structures of model compounds. In making this type of assignment for non-stoichiometric compounds, there is always a difficulty in determining the total number of ions per unit cell (or looked at another way, the vacancy concentration per unit cell). In systems where single crystals are available, this can be resolved by comparing the experimental density and the density calculated for the various proposed unit cell contents. However, the very small enamel apatite crystals are embedded in a protein matrix, so the individual enamel apatite crystal densities cannot be measured. The resolution of this difficulty must rely on a structural assumption, in this case that all the phosphate sites are occupied. Making such an assumption, it is possible to calculate the density of the enamel apatite crystals, which in turn can be used to convert the mass fractions of apatite determined into volume fractions. Volume fraction is an important parameter for understanding transport processes and rates of chemical reaction in enamel, although measurements of tortuosity (a measure of how pore structure is interconnected) are also required.

Structures of model compounds

The hexagonal fluorapatite structure has been described many times (for example, Young & Brown 1982, Elliott 1994) and particularly well with the use of colour pictures by Beevers & McIntyre (1946). A prominent feature is that the PO_4^{3-} ions are approximately hexagonally close packed, which leads to the formation of channels through the structure; linear columns of F^- ions (each surrounded by three

Ca^{2+} ions) are placed into some of these channels, while columns of Ca^{2+} ions are placed in the remainder (Elliott 1973). The hexagonal packing of PO_4^{3-} ions is probably responsible for the stability of the apatite lattice, and hence the large numbers and variety of ionic substitutions make the study of apatites at times very challenging.

Fluorapatite has a hexagonal space group, $P6_3/m$, with lattice parameters $a = 9.367$ and $c = 6.884$ Å (Sudarsanan et al 1972). Pure hydroxyapatite, on the other hand, is only pseudohexagonal with a doubled b -axis ($a = 9.4214$ Å, $b = 2a$, $c = 6.8814$ Å, $\gamma = 120^\circ$) and monoclinic space group $P2_1/b$ (Elliott et al 1973). The doubled axis is associated with an ordered arrangement of the OH^- ions that only develops if the head-to-tail integrity of the OH OH OH OH columns is maintained. Thus, hydroxyapatite, with about 10% of the OH^- ions replaced by F^- ions, is hexagonal. Likewise, biological apatites also seem to be hexagonal, presumably because they are sufficiently 'impure' for the OH^- ion directions to be switched within columns.

Calcium-deficient apatites

Many hypotheses and structural models have been proposed to explain the often-made observation that precipitated apatitic calcium phosphates have X-ray diffraction patterns very similar to poorly crystallized hydroxyapatite, despite the fact that their Ca/P molar ratios can vary from less than 1.5 to greater than 1.67 (reviewed in Elliott 1994). Although these hypotheses include surface adsorption mechanisms and intercrystalline mixtures of octacalcium phosphate, as indicated earlier, only lattice substitutional models will be considered (Table 1); some of these have been extended to include carbonate apatites.

Evidence for these models comes mainly from chemical analyses and estimates of HPO_4^{2-} content from the pyrophosphate formed on heating. In addition, precipitated apatites generally have an a -axis that is 0.01–0.02 Å larger than high

Table 1 Selection of models of lattice substitutions in synthetic precipitated apatites

<i>Model</i>	<i>Limits</i>	<i>Reference</i>
$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$	$0 \leq x \leq 2$	von Kühl & Nebergall (1963)
$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}(\text{H}_2\text{O})_x$	$0 \leq x \leq 1$	Berry (1968)
$\text{Ca}_{10-x+u}(\text{PO}_4)_{6-x}(\text{HPO}_4)_x(\text{OH})_{2-x+u}$	$2-x+2u \leq 2$ and $0 \leq u \leq x/2$	Labarthe (1972)
$\text{Ca}_{10-x+y}(\text{CO}_3)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x+2y}$	$0 \leq x \leq 2$ and $2y \leq x$	von Kühl & Nebergall (1963)
$\text{Ca}_{10-x+u}(\text{PO}_4)_{6-x}(\text{CO}_3)_x(\text{H}_2\text{O})_x(\text{OH})_{2-x+2u}^a$	$0 \leq x \leq 2$ and $0 \leq 2u \leq x$	Bonel et al (1975)
$\text{Ca}_{10-x}\text{Na}_{2x/3}(\text{PO}_4)_{6-x}(\text{CO}_3)_x(\text{H}_2\text{O})_x(\text{OH})_{2-x/3}^b$	$0 \leq x \leq 3$	Bonel et al (1975)

^aWater lost below 400 °C was thought to occupy spaces formed by clustered vacant O (3), Ca (2) and OH sites.

^bWater thought to occupy tetrahedral site when PO_4 replaced by CO_3 .

temperature hydroxyapatite (Trautz 1955). Trautz found that the a -axis parameter became slightly larger, and the c -axis perhaps slightly smaller, as the Ca/P ratio decreased, but actual values were more variable than could be explained by changes in Ca/P ratios or experimental errors. He thought that factors besides the Ca/P ratio, particularly coprecipitated H_2O , influenced the axial parameters. Compared with hydroxyapatite, calcium-deficient hydroxyapatites have weaker OH^- infrared bands at 3672 and 630 cm^{-1} ; a stronger HPO_4 band at approximately 870 cm^{-1} ; less OH^- X-ray scattering, but an increased total X-ray scattering on the c -axis; and, together with dental enamel, show contractions in the a -axis on heating to about 300°C attributed to a loss of HPO_4 and/or H_2O from the structure (reviewed in Elliott 1994). These observations are consistent with the general principles of formulae in Table 1.

Structure of carbonate apatites

There has been much controversy about the relation between the CO_3^{2-} ion and the apatite lattice in mineral, synthetic and biological apatites, although there is now general agreement about many aspects, as summarized below. Further details and literature references can be found in a recent review (Elliott 1994).

Substitution for PO_4 (B-type substitution). In the well-crystallized carbonate-containing fluorapatite mineral francolite, CO_3^{2-} ions replace PO_4^{3-} ions. This finding is based on chemical analyses, a systematic reduction in the a -axis as the CO_3 content increases, and polarized infrared measurements which show that the CO_3^{2-} ion is oriented so that it occupies the sloping face (with respect to the basal plane) of a PO_4 site. It is also generally accepted that the same replacement takes place in precipitated carbonate-containing apatites, although the location of the ion may not be so well defined.

Substitution for OH (A-type substitution). The other form of CO_3 substitution in the apatite lattice is the replacement of two OH^- ions by one CO_3^{2-} ion. Evidence for this is a systematic increase in the a -axis with CO_3 content, chemical analyses and polarized infrared measurements that show that the plane of the CO_3^{2-} ion is nearly parallel with the c -axis, as is required by steric considerations. Although substantial replacement of OH^- ions requires very dry conditions at 900°C , there is evidence from infrared studies that a limited replacement can take place in precipitated carbonate-containing apatites.

Water in apatites

Some of the structural models in Table 1 propose that H_2O is located in the c -axis channel sites, either filling all, or some, of the sites left vacant by the loss of OH^-

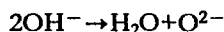
ions. In addition, suggestions have been made that H_2O might occupy vacant calcium sites (Rowles 1964, McConnell 1970) and accompany CO_3^{2-} ions (Table 1). Lattice parameter changes on heating are consistent with H_2O in the lattice (see above).

Chemical composition of enamel apatite

Ideally, the starting point for a discussion of the structure of the apatitic phase should be its chemical composition in native enamel. Although methods have been described for the removal of the protein phase (see Zahradnik & Moreno 1975 for references), there is inevitably the possibility that the inorganic phase will be modified, particularly by the loss of H_2O or CO_3 . Thus, in practice, the starting point must be a chemical analysis of whole, native enamel.

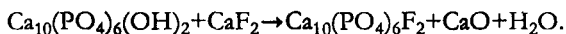
Oxygen, hydrogen and water

In studies of minerals, oxygen is rarely analysed for directly, although accurate determinations can be made by activation with 14 MeV neutrons using the reaction $^{16}\text{O}(\text{n,p})^{16}\text{N}$ in which γ -rays of 6.1 and 7.1 MeV are produced (Volborth et al 1975). Normally, the oxygen content is deduced from the composition expressed in terms of oxides, including the constitutional H_2O . The constitutional H_2O includes all the lattice H_2O of hydration and combined H_2O , e.g. as OH^- ions, the latter normally taken as the H_2O driven off above 100°C , by strong heating, via the reaction

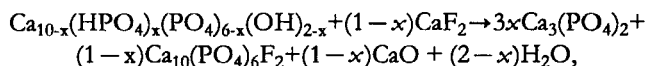


until no OH^- or other protonated species remain. After subtraction of the oxygen equivalent of the F^- and Cl^- contents, the sum of the percentages of oxides and constitutional H_2O should equal 100%, provided the oxidation states of the cations have been correctly assigned, which is unlikely to be a problem with biological apatites. Many analyses expressed in this form for apatites are to be found in the mineralogical literature (Cruft et al 1965, McConnell 1973).

Constitutional H_2O in apatitic systems cannot be determined from the H_2O evolved solely by strong heating because hydroxyapatite is stable to at least 1400°C , unless in a very dry atmosphere (Riboud 1973). Cruft et al (1965) have discussed suitable alternative methods for determining total hydrogen (equivalent to the constitutional H_2O) in apatites. They recommended Penfield's method in which the H_2O evolved on fusion with a mixture of silica, lead chromate, litharge and calcium carbonate is collected and weighed. Alternatively, for many samples, including biological apatites, Wallaey's (1952) method might be adapted. In this, the sample is ground with finely divided CaF_2 and the change in mass measured in the reaction



For the first model in Table 1, the reaction would be:



which takes place on heating for some hours at 800 °C. A substantial improvement to this scheme would be to collect and weigh the evolved H₂O, thus avoiding corrections for changes in weight due to CO₂ loss.

In enamel apatites, in order to gain information about the H₂O content, most previous workers have measured the weight loss as a function of temperature. After correction for the decomposition of protein and CO₃, the loss at the highest temperature (typically 900–1400 °C) has been either explicitly or implicitly equated with the total H₂O content (H₂O in the organic phase + constitutional H₂O in the apatite phase). There are two difficulties with this procedure. The first is that additional errors are introduced because the weight losses have to be corrected for the concomitant loss of protein and CO₃. The second major problem is the one referred to above in connection with mineral apatites, namely that there will always be an uncertain amount of constitutional H₂O remaining, even after strong heating; further, the oxyhydroxyapatite formed is very likely to rehydrate on cooling. Alternatively, the total H₂O could be deduced by difference because the total percentages expressed as oxides should add up to 100% (with a correction for the protein); however, errors in the analyses would contribute to the error in total H₂O content. As a result of the above considerations, it is unlikely that any of the published measurements of H₂O in enamel accurately give the sum of the H₂O in the organic phase plus the constitutional H₂O of the apatite phase.

Even if the total H₂O content is correctly found, there still remains the problem of division between the organic and apatitic phases. It will not be possible to make a clear distinction on the basis of measurements of H₂O evolved at different temperatures, although changes in lattice parameters could indicate that apatitic constitutional H₂O was being lost. In practice, the best that can be done is to make some assumption that H₂O evolved below a certain temperature, say 110 °C, is associated with the non-apatitic phase, and the rest with the apatite.

The total hydrogen content of enamel can also be measured by wide line proton nuclear magnetic resonance, since the integral of a proton resonance line is proportional to the mass of hydrogen associated with it. Furthermore, the line width is inversely proportional to the rotational freedom of the atoms. In nuclear magnetic resonance H₂O equilibration studies at 100 MHz, Dibdin (1972) found that human enamel equilibrated at 97% relative humidity at 20 °C for three days showed two lines, a narrow line of 150 mG, which represented between 0.8 and 1 wt. % of loosely bound H₂O, and a broad line of 1 G, which represented about 1.6 to 2 wt. % of strongly bound H₂O or 3–3.8 wt. % OH⁻ ions. The narrow line was essentially lost after keeping the enamel at 4% relative humidity at 20 °C for 20 h. The easily accessible H₂O can also be estimated from H₂O absorption isotherms (Zahradnik & Moreno

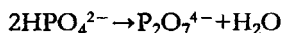
1975, Dibdin & Poole 1982) and reaction of enamel with the Karl Fischer reagent (Bonte et al 1988). In experiments at 20 °C, Dibdin (1972) found that whole human enamel gave a H₂O content of 1.15 wt. % for isotherms measured from vacuum to 100% relative humidity (outer enamel was approximately 0.9 wt. %). Bonte et al (1988) analysed human enamel fragments (30–100 mg) prepared from teeth that had been equilibrated at 20 °C and atmospheric relative humidity for 24 h and crushed in an agate mortar. Another set of enamel samples were stored over 0.5 mol/l NaCl for up to 45 days. The first set of samples had a mean H₂O content of about 1 wt. % (range 0.56–1.48), but some samples from the second set had H₂O contents up to 3.5 wt. %.

Calcium, magnesium and sodium

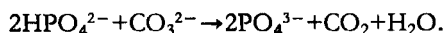
There is no difficulty in analysing calcium, magnesium and sodium and assigning at least the calcium and sodium to the apatitic phase (calcium replacement by sodium is well known in minerals). Even though the radius of Mg²⁺ is much smaller than Ca²⁺ (0.72 versus 1.00 Å), there seems little doubt that the small amount of replacement of calcium by magnesium required to accommodate the amount of magnesium found in enamel is within the limited substitutional range that is possible, although as mentioned earlier, it has been suggested that magnesium is present in a separate phase.

Phosphorus

Virtually all the phosphorus in native enamel is present as PO₄³⁻ or HPO₄²⁻ associated with the apatite phase. Unlike the situation with CO₃²⁻ and HCO₃⁻ (see below), attempts have been made to determine the fraction of orthophosphate that is protonated. Two methods are most frequently used: a chemical and an infrared method. The chemical method (Gee & Dietz 1955) uses the formation of pyrophosphate on heating at a temperature between 400 to 700 °C through the condensation reaction



and subsequent analysis of pyrophosphate. However, there are doubts about the quantitation of this reaction because of reduced pyrophosphate yield, either through loss via formation of β -Ca₃(PO₄)₂ or more particularly, through loss of HPO₄ via reaction with CO₃²⁻:



In the infrared method, the intensity of the absorption of the HPO_4 band at 875 cm^{-1} is measured. If CO_3 is present, a correction has to be applied, as it absorbs in the same region.

Arends & Davidson (1975) found the HPO_4^{2-} content of sound human enamel to be 5.2 ± 2.4 wt. % using the infrared method and 4 ± 0.5 wt. % from pyrophosphate formed on heating. Taking the weight per cent HPO_4^{2-} as 5 wt. %, this means that about 5% of the phosphate ions carry protons (the figure used in Table 3).

Carbon

Carbon is present in both the apatite and organic phase, but that in the apatite phase (present as CO_3^{2-} or HCO_3^-) can be estimated by standard methods, although the relative amounts of each cannot be determined. Infrared studies show that about 11% of the CO_3^{2-} ions are in the c -axis channels in dental enamel (the figure used in Table 3 [Elliott et al 1985]). In heated enamel, some of the apatitic carbon will also be present as molecular CO_2 .

Nitrogen

Total nitrogen determined in native enamel can be ascribed to the organic phase as protein but in heated enamel, although most protein is easily lost, some produces NCO^- and NCN^{2-} ions associated with the apatite phase. The N weight per cent has been converted to protein weight per cent by multiplication by the conventional factor of 6.25 (as in Little & Casciani 1966). However, since the amino acid composition of mature enamel is known (Table 32.5 in Cole & Eastoe 1988, assuming 20% H_2O soluble and 80% insoluble ribbons), a more appropriate factor can be calculated, which is 6.67. Thus, estimates of protein weight per cent based on the conventional factor will be 6.7% too low. This is clearly significant if the protein content is used in estimates of the apatite weight per cent. The factor to multiply the N weight per cent by to obtain the H weight per cent of the protein can also be estimated from the same amino acid analyses, and is 0.445.

Structural models for enamel apatite unit cell

Previous models

As mentioned earlier, there is little direct structural information on enamel, although Young & Mackie (1980), in a combined X-ray and neutron diffraction study, concluded that there were differences between the structures of hydroxyapatites and enamel apatite in the distribution of the scattering density along the c -axis, including differences between the X-ray and neutron estimations that would accommodate H_2O in some orientational disorder centred between $z = 0.11$ to 0.14 , as well as the approximate 0.3 wt. % chlorine that enamel contains. Other differences have been noted above. Several formulae for enamel apatite have been published (Table 2), but

Table 2 Some proposed models for the formula of enamel apatite

<i>Model</i>	<i>Reference</i>
$\text{Ca}_{9.48}\text{Mg}_{0.18}\text{Na}_{0.11}(\text{PO}_4)_{5.67}(\text{CO}_3)_{0.45}(\text{OH})_{1.54}(\text{H}_2\text{O})_{0.46}$	Hendricks & Hill (1942)
$\text{Ca}_{9.26}(\text{HPO}_4)_{0.22}(\text{CO}_3)_{0.5}(\text{PO}_4)_{5.63}(\text{OH})_{1.26}^{\text{a}}$	Aoba & Moreno (1992)
$\text{Ca}_{9.23}\text{Na}_{0.26}\text{K}_{0.03}(\text{PO}_4)_{5.53}(\text{CO}_3)_{0.47}(\text{OH})_{1.15}\text{Cl}_{0.06}\text{F}_{0.01}^{\text{b}}$	Driessens (1978)

^aLate mature porcine enamel.

^bWith vacancies at unfilled Ca, PO₄ and OH sites.

many are inconsistent in some respect with likely unit cell contents derived from the above discussions on model compounds.

Models deduced from chemical analyses

Of the published chemical analyses which might be used to illustrate how unit cell contents, apatite fraction and density of enamel apatite can be calculated, that of Little & Casciani (1966) (Table 3) was chosen because it includes information on the weight loss on heating to 1400 °C. This analysis is expressed in terms of species to be located in sites in the apatite cell, which is more appropriate than using oxides. The procedure for each model is that the molar ratios of the species (excluding H₂O because it cannot be assigned to sites at this stage) are first calculated and multiplied by a factor so that all the PO₄ sites are filled with the ions allocated to this site. The OH⁻ content is then calculated from the requirement for charge balance. The results are given in the 'moles' column. Water can then be assigned to the vacancies in Ca, PO₄ and OH⁻ sites, although only those in the most probable, i.e. the OH site vacancies, are included in subsequent calculations. The weight per cent of each protonated species can then be calculated from its moles per unit cell. To derive the weight per cent of apatite in enamel, the weight per cent of protonated species are summed with the weight per cent of the non-protonated species found by Little & Casciani (1966). The relative atomic masses of the unit cell constituents can then be summed (listed as the formula weight) and multiplied by the atomic mass unit (1.660 × 10⁻²⁷ kg) to give the mass of the unit cell. This, divided by the unit cell volume (530.92 Å), gives the density of the enamel apatite crystals.

The models in Table 3 are arranged in order of increasing complexity. Model 1, with only PO₄³⁻ ions in PO₄ sites leaves no space for the CO₃²⁻ ion, has fewest calcium site vacancies and has the highest density. Model 4 takes into account HPO₄²⁻ ions and CO₃²⁻ in the *c*-axis channels, and it is likely to be closest to reality. Note that the addition of HPO₄²⁻ makes a significant difference to the composition in these channels.

Table 3 Various models for enamel apatite based on analyses of Little & Casciani (1966), calculated densities and lattice parameters $a = 9.441$ and $c = 6.878$ Å (Young & Mackie 1980)

	<i>Model 1</i> ^b		<i>Model 2</i> ^c		<i>Model 3</i> ^d		<i>Model 4</i> ^e		
<i>Unit cell species</i>	<i>wt. (%)</i> ^a	<i>moles</i>	<i>wt. %</i>	<i>moles</i>	<i>wt. %</i>	<i>moles</i>	<i>wt. %</i>	<i>moles</i>	<i>wt. %</i>
Calcium ion sites:									
Ca ²⁺	36.42	9.502		8.783		8.856		8.856	
Mg ²⁺	0.22	0.094		0.087		0.088		0.088	
Na ⁺	0.70	0.314		0.290		0.292		0.292	
K ⁺	0.03	0.010		0.010		0.010		0.010	
vacancy/H ₂ O		0.080	(0.14)	0.830	(1.55)	0.754	(1.40)	0.754	(1.40)
Phosphate ion sites:									
PO ₄ ³⁻	54.58	6		5.546		5.592		5.312	51.78
HPO ₄ ²⁻								0.280	2.76
CO ₃ ^{2-f}	2.79	0.492		0.454		0.407	2.51	0.407	2.51
H ₂ O/vacancy		0.492	(0.85)	0.454	(0.85)	0.407	(0.75)	0.407	(0.75)
Hydroxyl ion sites:									
OH ⁻		0.448	0.73	0.417	0.73	0.422	0.74	0.702	1.40
Cl ^{-g}	0.30	0.084		0.077		0.078		0.078	
H ₂ O/vacancy		1.468	2.53	1.506	2.81	1.400	2.59	1.120	2.07
CO ₃ ²⁻						0.050	0.31	0.050	0.31
Water:									
H ₂ O 24–100 °C	1.00 ^f								
H ₂ O 100–900 °C ^g	1.41								
H ₂ O 900–1400 °C ^g	3.54								
apatitic constitutional H ₂ O	3.89 ^h	1.692	2.91	1.714	3.19	1.611	2.98	1.611	2.98
Total apatite ⁱ	99.52 ^j		98.30		98.58		98.37		98.28
Formula wt. without H ₂ O		1000.67		924.96		932.63		939.37	
Density gcm ⁻³ without H ₂ O			3.129		2.892		2.916		2.937
Formula wt. with H ₂ O		1027.11		967.05		971.43		957.82	
Density gcm ⁻³ with H ₂ O			3.211		3.023		3.037		2.993

^aLittle & Casciani (1966) with CO₂ converted to CO₃, and Cl from Bowes & Murray (1935).^bPO₄ sites filled with 6PO₄.^cPO₄ sites filled with 6(PO₄+CO₃).^dPO₄ sites filled with 6(PO₄+0.89CO₃) and 0.11CO₃ in OH sites.^ePO₄ sites filled with 6(0.95PO₄+0.05HPO₄+0.89CO₃) and 0.11CO₃ in OH sites.^fExcluded from total by Little & Casciani (1966) and in this Table.^gLess CO₂ and H₂O. Little & Casciani (1966) also give details for smaller intervals.^hThis is the present author's estimate from taking a quarter of the 100–900 °C H₂O and all of the 900–1400 °C H₂O.ⁱTotal of non-hydrogenated species from Little & Casciani (1966) plus unbracketed hydrogenated species from model.^jFrom Little & Casciani (1966) with CO₃ reported as CO₂ and H₂O from 100–1400 °C. Such a sum is meaningless as some analyses are expressed as ions and some as neutral species.

With H_2O restricted to the c -axis channels, none of the models can accommodate the likely amount of constitutional H_2O , not even the H_2O evolved between 900 and 1400 °C. Either the estimate of constitutional H_2O is too high (for example, if substantial amounts were strongly bound to the apatite crystal surfaces) or H_2O is located in additional lattice sites, as proposed in the models of Bonel et al (1975) (Table 1). Another check on the validity of the estimate of constitutional H_2O can be obtained from the weight per cent of apatite in Model 4. Subtraction of this from 100% gives the organic phase as 1.72 wt. %. The weight per cent protein in enamel is 0.056% (N weight per cent from Little & Casciani 1966) multiplied by 6.67 (see above), which is 0.37 wt. %. Thus, 1.35 wt. % of enamel is accounted for by H_2O in the organic phase, a figure not out of line with direct non-thermal estimates. Added to the constitutional H_2O in Model 4, this gives the total H_2O as 4.20 wt. %, which is somewhat lower than the H_2O lost between 100 and 1400 °C (4.95 wt. %), itself a minimum estimate. Barring analytical errors, this discrepancy again suggests that H_2O is not fully accounted for in the model.

Determination of volume fraction of apatite in enamel

Continuing the two-phase model for enamel, if the densities of the enamel apatite, organic phase and whole enamel are ρ_a , ρ_o and ρ_e , respectively, and the mass and volume fractions of apatite are m and v , respectively, then v can be determined from any two densities via one of the equations:

$$\begin{aligned} v &= m\rho_e/\rho_a; \\ v &= 1 - (1 - m)\rho_e/\rho_o; \text{ or} \\ v &= \rho_o/(\rho_a - \rho_a m + \rho_o m). \end{aligned}$$

The first equation is unsuitable as ρ_e and ρ_a are so similar (see below) that any error in either would have a disproportionately large effect on v . It is better to use either of the last two equations. Taking $\rho_e = 2.95 \text{ g cm}^{-3}$ (Weatherell et al 1967), $\rho_o = 1.05 \text{ g cm}^{-3}$ (estimated for 21 wt. % protein of density 1.3 g cm^{-3}), $\rho_a = 2.99 \text{ g cm}^{-3}$ (Table 3) and $m = 0.983$ (Table 3), the second and third equations give $v = 0.952$ and $v = 0.969$, respectively. There is no reason to accept one value over the other, so the average gives the apatite content of enamel as about 96% vol.

Conclusions

The apatite concentrations in enamel of 98 wt. % and 96 vol. % deduced here are significantly higher than the 95 wt. % and 87 vol. % usually quoted. The latter seem to originate from Brudevold & Söremark (1967), who noted the difficulty in determining the H_2O content and suggested that a significant amount of their estimate of 4% wt. might be associated with the mineral in some unknown manner. In fact, explicit inclusion here of constitutional H_2O bound as OH^- , HPO_4^{2-} and

molecular H_2O is the main reason for the higher weight per cent. In addition to an increased weight per cent of apatite, the volume per cent is also increased over Brudevold & Söremark's value by the use of 2.99 g cm^{-3} for the enamel apatite density in the conversion. This figure is more appropriate than 3.15 g cm^{-3} , the density of hydroxyapatite used by Brudevold & Söremark, because it takes into account the known expanded lattice of enamel apatite and its likely calcium vacancies. If the densities (ρ_a , ρ_c) of 3.15 and 2.95 g cm^{-3} had been used to convert the enamel apatite weight per cent of 98.3% using equation 1 above, the result would have been 92.0 vol. %, instead of 96.9 vol. %. It is clearly important to obtain an independent measurement of the volume per cent of the organic phase. This might be done either by direct estimate of the space between the apatite crystals from electron micrographs or by measurement of the uptake of a suitable species into the organic phase chosen not to react with the apatite phase.

Further investigations of the unit cell composition, and the weight per cent and volume per cent of enamel apatite require more detailed analyses, particularly of the constitutional H_2O for the reasons outlined above. As the H_2O in the protein phase is rather labile, the starting tissue should be equilibrated at a well-defined temperature and relative humidity (for example, 20°C and 97% relative humidity). Furthermore, it would be advantageous if there were nuclear magnetic resonance, infrared, H_2O absorption, lattice parameter and density studies on the same sample. Preferably, specific regions of enamel should be studied as the lattice parameters (Sakae 1988) and composition vary with distance from the enamel surface; this would give information on regional changes in mineral weight and volume per cent, which are important in models of the caries process. It might be possible to obtain independent measures of these changes from quantitative microradiography and direct estimation of the space between crystals from electron microscope photographs.

Acknowledgement

This research is supported by the Medical Research Council Grant Number G9505593-MA.

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DISCUSSION

Oldak: You demonstrated some of the differences between enamel crystals and synthetic apatite crystals, including differences in cell dimension, stoichiometry and particularly crystal density. Have you considered that the presence of protein inside enamel crystals may be the factor causing some of these differences?

Elliott: Frank et al (1960) proposed that proteins may be present inside crystals, but there is no direct evidence for this. Double salts of octacalcium phosphate dicarboxylic acids are also present (Monma 1984).

Oldak: There is solid evidence for the presence of intracrystalline proteins in some calcitic skeletal elements such as sea urchin and mollusc shells, and their location suggested that they play important roles in biogenic crystal formation. In addition, to some extent they determine crystallographic and textural properties of the biological mineral (Berman et al 1993).

Elliott: But there is no evidence for this in the apatite system.

Robinson: I would like to ask you about the determination of volumes. We did some mapping of mature permanent enamel sections by determining the physical density using a density-immersion technique (Robinson et al 1971). We found that the volume of mineral was 85–90%. In order to check that porosity was not influencing the volume measurements, we performed the analysis on embedded sections infiltrated with methyl methacrylate. This analysis showed that there were gradations in enamel density from the enamel surface towards the dentine. Low density areas contained relatively large amounts of protein.

Elliott: The volume per cent of enamel mineral in whole enamel calculated in my paper was based on chemical analyses and lattice parameter measurements, which I

believe is a sounder way than one that assumes that the enamel crystals have the density of pure hydroxyapatite. However, it would be useful to obtain an independent check of the result of this calculation.

Deutsch: What is your opinion of the quoted transient mineral phases leading to hydroxyapatite formation, and do they exist in enamel?

Elliott: Octacalcium phosphate is the most likely transient phase. The main evidence for this is the flattened hexagonal cross-section of enamel crystals. However, I am not sure whether transient phases exist or not.

Mann: If most of the water is contained within the octacalcium phosphate phase and not within the apatite structure how would this affect your calculations?

Elliott: If there were an octacalcium phosphate phase, its water content would affect the calculation. However, to my knowledge, no one has proposed that octacalcium phosphate is present in mature enamel; I assumed a single apatitic phase in my calculations.

Diekwisch: Mature enamel doesn't contain much water but during development the water content does increase (Robinson et al 1988). Can you comment on this?

Elliott: Enamel is relatively heterogeneous. As mentioned in my paper, the lattice parameters and water absorption isotherms vary, so it is possible that the water content of the organic phase also varies.

Robinson: Little & Casciani (1966) performed some experiments in which they had driven off 0.6–1% of the water at about 100 °C, which is the middle temperature you quoted. Using nuclear magnetic resonance imaging, they observed that the remaining water was 'highly restricted, freely tumbling, but caged'. What did they mean by this?

Elliott: As I discuss in my paper, Dibdin (1972) reported a narrow and wide proton resonance line from enamel using a sealed system under controlled humidity. These were ascribed to mobile water and more strongly bound protons, respectively. The narrow line, which seemed to correspond to the narrow line reported earlier by Myers (1965) and Little & Casciani (1966), was lost on reducing the relative humidity to 4%. This contradicted the work of earlier researchers, who reported that the narrow line had unusual stability against drying, even at high temperatures. It is possible that the narrow line derived from water picked up whilst the enamel was transferred to the spectrometer.

Snead: Hal Slavkin, in his introduction, commented that many of us have entered the field of enamel from different perspectives. I am interested in how the organic phase influences the inorganic phase. How do the protein aggregates bring about compositional changes in crystal structure?

Elliott: I do not know the answer, but my calculations provide a better estimate of the volume occupied by the organic material in the enamel and hence how close the protein molecules are to each other: this could well be relevant to the eventual answer to your question.

Fincham: Your calculations are based on human enamel, but are there variations in the nature of the mineral content between species?

Elliott: Leicester (1949) has made an extensive compilation of early analyses from the literature. These indicate that rat upper incisor enamel has a higher iron content than human enamel, that teeth from sharks and many other animals living in the sea contain 1–2% fluorine, and that the dentine magnesium content of many animals is much higher than in humans. More recent references to species variations of biological apatites can be found in Elliott (1994).

Fincham: It's interesting to wonder about what might be the basis of such variation.

Slavkin: The question of whether genotype or environmental factors control the ion content of developing teeth is intriguing. We know that elevated levels of fluoride produce human dental enamel fluorosis with significant pathology of enamel, but we do not clearly understand if and how genes control the ion content of enamel. In my memory, it was the pioneering research by the late S. Suga who tested the hypothesis that the genotype of fresh and salt water fish controlled fluoride content, rather than it being a strict reflection of the concentration of fluoride in the water. S. Suga and his colleagues in Japan provided a substantial volume of evidence to support their hypothesis.

Deutsch: In 1985 I spent a short sabbatical in the lab of the late S. Suga. We were trying to study the problem of how the enameloid of the teeth of some sharks contains very high concentrations of fluoride (reported to be as high as 38 000 ppm) despite the fact that the concentration in sea water is only around 1 ppm. I dissected the layer of cells covering the enameloid surface and found the concentration of this layer to contain relatively high concentrations of fluoride. However, because the enameloid extracellular tissue (which contains large amounts of fluoride) is soft during formation and early maturation stages, the relatively high concentration of fluoride found in the 'enameloid organ' could have been due to contamination during dissection by the underlying soft enameloid. Nevertheless, it could still be due to some concentrating mechanism existing in shark enameloid-producing cells.

Snead: Changing the physical composition of a protein may change its binding capabilities. For example, Aoba et al (1989) have shown that the teleopeptide at the C-terminus of amelogenin has a capacity to bind to hydroxyapatite, but when the teleopeptide is cleaved, the processed amelogenin no longer binds. The typical hydroxyapatite used in column chromatography is synthetic, but do hydroxyapatites that have different chemical substitutions have the same binding capacities?

Elliott: The answer is almost certainly no. For example, apatites are used as catalysts for the dehydrogenation of alcohols and it has been shown that changing from calcium to strontium hydroxyapatite affects the reaction rate (Joris & Amberg 1971). It would also be interesting if we could get a handle on the surface structure of these apatite crystals.

Robinson: The fluoridated apatites tend to bind proteins rather more readily than non-fluoridated apatites, although I'm not referring to fluorapatite here because that's a rather special case. pH is another complicating factor. Aoba's experiments were all performed at pH 6.0, which is non-biological, but he has also performed some at pH 7.8 (Aoba et al 1987). The surface chemistry is critical, and therefore the

environment in which the experiments are performed is extremely important—small changes in pH and local ionic strength will not only affect whether a protein binds but also what state the protein is in. Many of these experiments are conducted under non-biological conditions, which means that the same result may not necessarily apply *in vivo*.

Slavkin: Amelogenin is a hydrophobic, lipid-like protein and may be a candidate for removing water during the period of crystal growth. Is it possible to determine how many amelogenin molecules per unit area would be required to remove sufficient water to facilitate the growth of hydroxyapatite?

Elliot: One ought to be able to calculate how many amelogenin molecules there are per unit area of hydroxyapatite and what the concentration is in this fluid phase.

Snead: This does assume though that one has some idea of the packing order of amelogenins. We are talking about 'nanospheres', which assumes one particular diameter, but the problem is that there are no structural data and there is a lack of homogeneity in the amelogenin isoforms that are expressed.

Veis: It may be possible to use techniques such as Fourier transform infrared spectroscopy to determine the relative protein content of the enamel crystals. Proteins show a strong absorbance in the region of 1650 cm^{-1} , so it could be done in a fairly quantitative fashion.

Rosenbloom: But this analysis may not take into account local variations in the relative amounts of amelogenin splice variants, for example.

Mann: Also, proteins could just be absorbed at specific sites where there are kinks or defects. Therefore, at these sites, relatively low amounts of the proteins may have a dramatic effect on preventing the lateral growth of the crystals.

Deutsch: Binding of an acidic protein to a hydroxyapatite crystal is not necessarily a specific reaction. Addadi et al (1992) have developed a system in which they add proteins during crystal formation. If the protein is specific, it binds to crystal motifs and inhibits a particular crystal face from growing.

Butler: The results of Addadi et al (1992), Boskey et al (1990) and Hunter & Goldberg (1993) suggest specificity with regard to the role of proteins in nucleation of mineralization, whereas as the influence on crystal growth is less specific.

Simmer: What do you mean by specificity in that context?

Butler: In terms of crystal growth in *in vitro* experiments, a number of acidic proteins, such as osteopontin, change the growth rate of hydroxyapatite crystals. Osteopontin will not, however, initiate crystal growth, whereas bone sialoprotein will. Therefore, there is more specificity relative to the initiation of growth than to the control of crystal growth.

Simmer: Are you suggesting that the proteins that control crystal growth, which may repeatedly associate with the crystallites in one or a small number of orientation(s), bind non-specifically because they are unable to initiate crystal growth *in vitro*?

Butler: I am suggesting that several proteins which are acidic and phosphorylated bind to surfaces of growing apatite crystals in an orientation that affects the growing surface, slowing crystal growth. This binding is non-specific in the sense that a number of proteins are effective at this type of interaction.

Oldak: Highly specific interactions between a crystal surface and protein functional groups can only occur if all the requirements for a perfect match between the two are met. For example, the interaction between phosphophoryn and the (010) faces of octacalcium phosphate (Furedi-Milhofer et al 1994, Moradian-Oldak 1992) is influenced by calcium-calcium complexing distance, stereochemistry of the phosphate groups emerging from the interacting crystal face (its orientation) and the structured functional group of the protein, in this case the phosphate groups of phosphophoryn. A lower level of specificity or a less perfect match can also affect crystal growth. In this case not all the factors mentioned above are involved in the interaction, and this was seen (*in vitro*) in the case of a carboxylate-rich protein interacting with the (100) faces of octacalcium phosphate. Different degrees of specificity can also be applied in *in vivo* systems, in terms of nucleation and influences on crystal shape during crystal growth. In order to obtain oriented crystal nucleation, at least in the case of calcite-containing systems, some level of specificity in interaction between the protein nucleator and the nucleating crystals is required (Addadi et al 1987). I am not sure whether this would be required for the oriented growth of apatite-containing systems, and particularly for enamel.

Veis: Nucleating mineralization and regulating crystal growth are two different processes. In one case, protein orientation stimulates the arrangement of ions for nucleation and growth. On the other hand, the binding of a protein molecule on specific crystal faces may regulate the rate of growth.

Oldak: Yes, but what I would like to emphasize is that specific interactions between protein matrix and nucleating crystals may not be required for obtaining the oriented growth of enamel apatite crystals. Some other factors, including 'calcium flux' by the ameloblasts, may well be involved.

Boyd: Has anyone taken growing enamel crystals and looked at their behaviour in a system without protein?

Robinson: Yes. They retain the same cross-sectional hexagonal morphology. However, the hexagons tend to become more flattened in the presence of a high level of carbonate.

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Molecular strategies of tooth enamel formation are highly conserved during vertebrate evolution

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Abstract. The vertebrate body plan is determined by a variety of morphoregulatory genes that are highly conserved throughout evolution. This review presents a phylogenetic analysis of selected molecular and morphological features in vertebrates with particular emphasis upon the phylogeny of tooth morphogenesis and enamel formation. Three lines of evidence support our hypothesis that the agnathans (e.g. hagfishes) are the most primitive extant vertebrates and that enamel gene products are highly conserved during vertebrate evolution. First, an antibody raised against the polypeptide produced by exon 4 of the mouse amelogenin gene recognizes proteins in hagfish, sharks, reptiles and mammals. Second, electron photomicrographic evidence suggests heterochronic shifts in the relative time and rate of enamel formation during vertebrate tooth evolution. Third, mRNA phenotyping suggests significant homology between amelogenin transcripts expressed in species of various vertebrate phyla including agnathans and mammals. These three lines of evidence indicate that amelogenin gene products are expressed in agnathan, reptilian and mammalian teeth.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 73-84

Recent discoveries provide clues to the molecular and morphological determinants that are highly conserved between invertebrates, early vertebrates and their modern relatives. The most intensively studied genes which control vertebrate development are those first isolated on the basis of their homology to conserved sequence motifs within genes (homeotic genes) that regulate *Drosophila melanogaster* development: homeobox-containing genes, paired box family genes and zinc finger-containing genes (Duboule & Morata 1994). These morphoregulatory genes participate both in the establishment of segmentation and in the specification of segment identity (see review by Krumlauf 1994). They also function during inductive epithelial-mesenchymal interactions associated with branchial arch, tooth and limb morphogenesis. Numerous gain- and loss-of-function experiments are providing important new advances towards understanding the molecular basis of

morphogenesis (Wolpert 1994, Kessler & Melton 1994). In terms of morphological features, recent advances in palaeontology are unravelling the fossil records and providing new interpretations for the relationships between early vertebrates and their modern relatives.

The first vertebrate arose approximately 550 million years ago in the Cambrian period, immediately following the enormous speciation within multicellular plants and animals (Gould 1989). Unfortunately, multicellular organisms (e.g. the extant *Amphioxus*, a cephalochordate) from that remarkable period of evolution have not left readily identifiable imprints within the fossil records. In fact, the oldest undeniably vertebrate remains do not appear until the Ordovician period, about 460 million years ago (Lovtrup 1977). These animals, known as ostracoderms, were small fish-like creatures that became extinct about 100 million years later. The ostracoderms gave rise to most of the extant vertebrates on the earth today including humans. Importantly, during the 100-million-year reign of the ostracoderms, a number of molecular and morphological features evolved including enamel, dentine, true bone, paired pectoral appendages and jaws (Forey & Janvier 1993, Smith & Hall 1990). The evolution of jaws (gnathostomes) also relates to the emergence of a number of complex vertebrate characteristics such as those associated with the maxillary and mandibular dentition.

This review presents a phylogenetic analysis of three lines of evidence that represent molecular and morphological vertebrate features, with particular emphasis upon the phylogeny of tooth morphogenesis and enamel formation. Evidence is presented which supports the hypothesis that the hagfish (agnathans) are the most primitive vertebrates known, extant or extinct. Further, the majority of ostracoderms were derived from the agnathic hagfishes or lampreys. Therefore, hagfishes and their ancestral derivatives provide opportunities to identify canonical molecular and morphological characteristics associated with the initiation and subsequent morphogenesis of gnathostomes and vertebrate-like tooth development. Features of extant hagfish such as the expression of amelogenin-like gene products without biomineralization and the sequence of tooth morphogenesis clearly relate to the history of gnathostomes. Finally, immunocytochemical and ultrastructural analyses of selected extant vertebrates provide additional lines of evidence for the conservation of several processes related to the initiation of enamel biomineralization during tooth development.

Experimental procedures

For electron microscopy, we fixed tissues in Karnovsky's fixative so that they could be osmicated and transferred immediately into a graded series of ethanols for dehydration. We then embedded the tissues in Epon 812 and cut 1 mm-thick sections for orientation and areas of initial enamel formation, and then 60–80 nm-thick sections using an ultramicrotome. As previously described (Diekwisch et al 1993, 1995), we used uranyl acetate and lead citrate to contrast the sections.

For immunohistochemistry, we fixed tooth organs of various vertebrates—including Pacific Hagfish (*Eptatretus stoutii*), Hornshark (*Heterodontus francisci*), Green Anolis (*Anolis carolinensis*) and mouse (*Mus musculus*)—with 10% buffered formalin, prior to decalcifying them in 4% EDTA and dehydrating them with a graded series of ethanols. We embedded the specimens in paraffin and cut 5 μ m-thick sections, which we probed with polyclonal primary antibodies (courtesy of J. Simmer and A. Fincham) raised against the polypeptide produced by exon 4 of the mouse amelogenin gene. Anti-rabbit IgG served as a secondary antibody. We detected the signals using aminoethyl carbazole as a substrate. In-between these procedures, we washed the slides in phosphate-buffered saline.

For mRNA phenotyping, we assayed RNA extract samples (Rappolee et al 1988) using 5' and 3' primers designed to include or exclude one or more of the seven exons that constitute the mouse amelogenin gene structure (see reviews by Fincham et al 1992, Zeichner-David et al 1995). We generated the sequences using reverse transcriptase PCR products amplified from young Pacific hagfish tooth RNA, sequenced them using an ABI Model 373A Sequencer (American Biosystems Inc., Foster City, CA, USA) and translated them for open reading frames in the convention of single letters representing each amino acid. We obtained alignments between selected vertebrate amelogenins using a basic alignment search tool (*BLAST*, Altschul et al 1990) and searching against the non-redundant protein database at the National Center for Biotechnology Information provided through the National Library of Medicine, National Institutes of Health, Bethesda, Maryland.

Heterochronic enamel development

During early mouse molar enamel formation, we observed the following three developmental events in terms of enamel biomineralization: (i) formation of amorphous precipitates at the dentine–enamel junction; (ii) formation of small clusters of initial short and randomly oriented crystals spatially separated from the adjacent dentine; and (iii) growth of long and parallel oriented crystals starting from the ameloblast cell membrane or aggregates of stippled materials (Fig. 1). These three developmental events characteristic of mouse initial enamel biomineralization resembled the patterns of initial enamel formation demonstrated in various vertebrates. We interpret these observations to indicate that during initial mouse enamel formation, developmental events characteristic of earlier vertebrate enameloid formation are recapitulated. These interpretations support aspects of Haeckel's biogenetic law (1866) of ontogeny replicating phylogeny. In order to explain species specificity and phylogenetic characteristics during enamel development, we postulate the presence of heterochronic shifts as suggested by de Beer (1937) between the transcriptional and translational events responsible for regulating enamel crystal formation in various species and phyla.

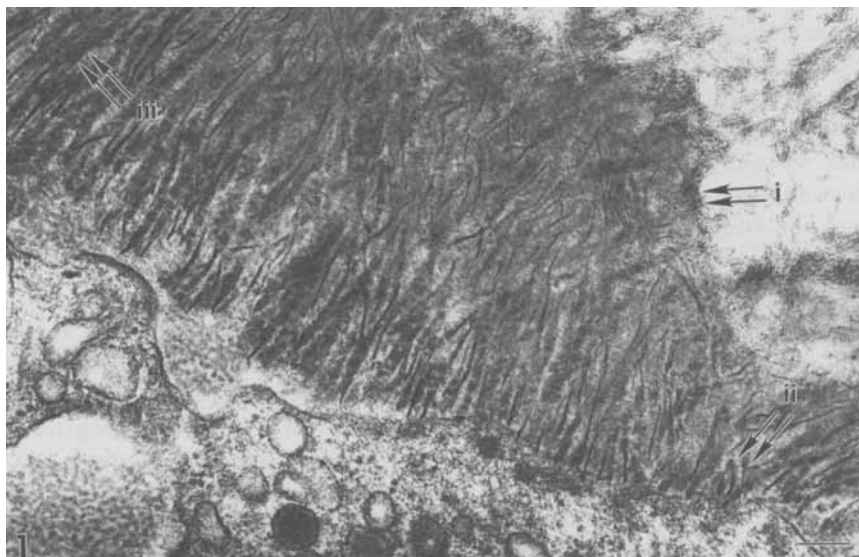
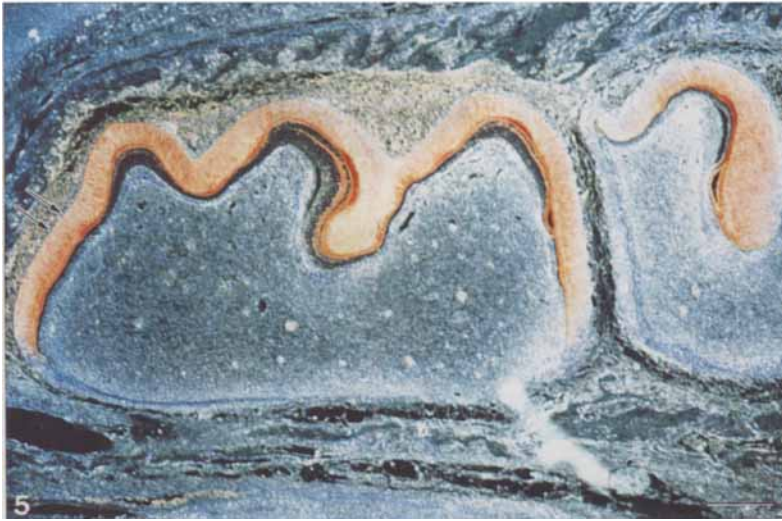


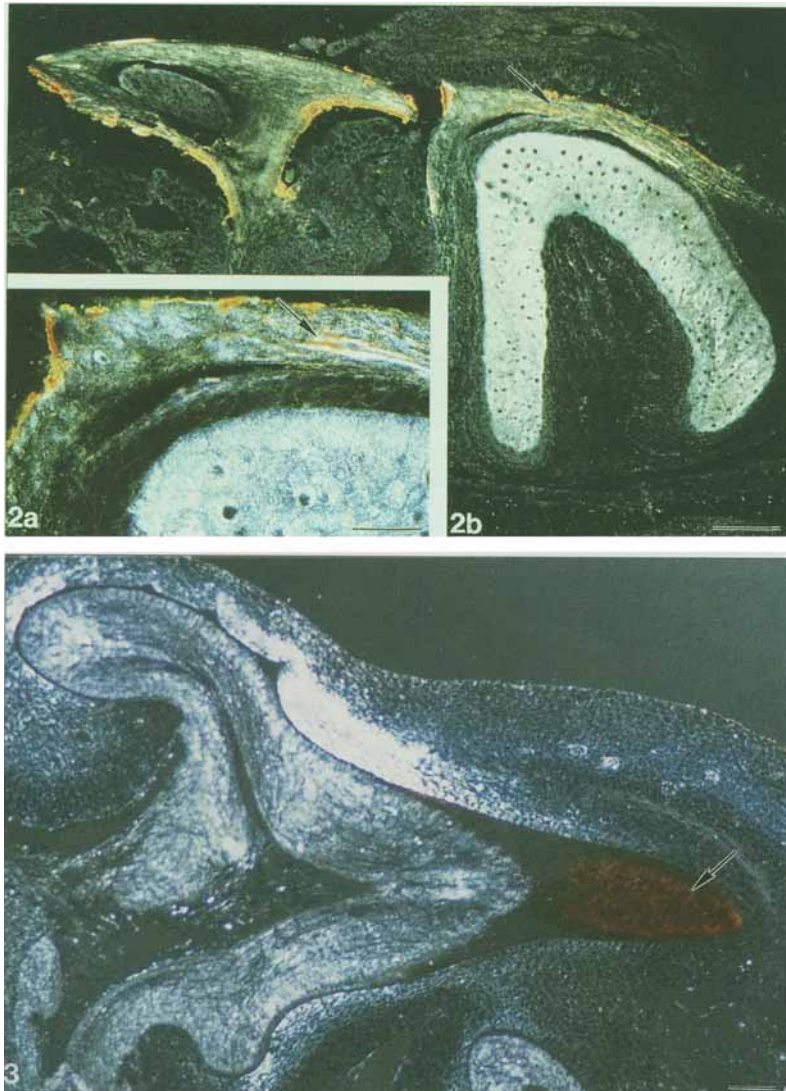
FIG. 1. Electron micrograph of developing mouse enamel demonstrating three events for enamel biomineralization: (i) formation of amorphous precipitates at the dentine–enamel junction; (ii) formation of small clusters of initial short and randomly oriented crystals spatially separated from the adjacent dentine; and (iii) growth of long and parallel oriented crystals starting from the ameloblast cell membrane or aggregates of stippled materials. Bar = 200 nm.

Amelogenin protein expression

In all the animals investigated, immunoreactions indicated the presence of amelogenin antigenic sites. We observed amelogenin immunopositive sites in ameloblasts and initial enamel/enameloid layers of the hagfish, shark, anolis and mouse (Figs 2–5). In the hagfish the amelogenin signals were confined to the tubular cells of the horn cap and the horn cap itself. (Fig. 2). Immunostaining localization was prominent on the surface of the horn cap of erupted as well as non-erupted tooth organs. In the shark a distinct portion at the tip of the crown was positively labelled (Fig. 3). In the anolis newly secreted enamel as well as the outer and inner enamel epithelium reacted with the antibody (Fig. 4). In the mouse the ameloblast cell layer and the enamel extracellular matrix demonstrated the presence of amelogenin epitopes (Fig. 5). These results support our hypothesis that amelogenins are present in the enamel/enameloid matrix of extant vertebrate species.



FIGS 2–5 Immunohistochemical labelling of tooth organs with an antibody against amelogenin exon 4 encoded polypeptide. The red immunostaining (arrow) indicates the localization of amelogenin epitopes both within ameloblasts and the adjacent developing extracellular enamel matrix. Amelogenin epitopes are detected in hagfish (Fig. 2a,b), shark (Fig. 3), anolis (Fig. 4) and mouse (Fig. 5). In the hagfish amelogenin immunostaining is localized both in the tubular cells and in the surface layer of the horn cap (arrows, Fig. 2a,b). In the shark a distinct portion at the tip of the crown is positively immunostained (arrow, Fig. 3).



In the anolis newly secreted enamel and both the outer and inner enamel epithelium are stained with the antibody (arrows, Fig. 4). In the mouse the ameloblast cell layer and the enamel extracellular matrix show amelogenin epitopes (arrows, Fig. 5). In all extant vertebrate species immunostaining is limited to the enamel organ. Bars = 100 μ m (Figs 2b, 3, 4, 5) and 50 μ m (Fig. 2a).

Vertebrate amelogenin mRNA sequences

Analyses of nucleic acid sequences demonstrated homology between mouse amelogenin and hagfish mRNA (Slavkin & Diekwisch 1996) (Fig. 6). We hypothesize that when gnathostome fish evolved from the more primitive agnathans (hagfishes and lampreys), multiple ancestral amelogenin genes were constituents of the agnathan genome and served multiple functions other than tooth-specific functions at this stage of vertebrate evolution.

Molecular phylogeny of amelogenin-like gene products

Amelogenins of higher vertebrates may have originated from a common ancestral gene (Herold et al 1980, 1989, Slavkin & Diekwisch 1996, Slavkin et al 1982, 1983, 1984, 1991, Thomson 1988). We have suggested, based upon preliminary immunological studies (Slavkin et al 1982, 1983, 1984, 1991), that the ancestral gene might be found within agnathans (hagfishes, *Eptatretus stoutii* and *Myxine glutinosa* L.). In previous investigations, we reported antigens within hagfish horny tooth coverings that were cross-reactive with polyclonal rabbit antibodies directed against the major mouse amelogenin (Slavkin et al 1982, 1983). More recently, using polyclonal rabbit antibodies directed against synthetic and recombinant mouse amelogenin polypeptides, we reported antigens within hagfish tooth pokal cells that were cross-reactive with these antibodies (Slavkin et al 1991). Importantly, the initial induction of an odontogenic placode and the subsequent dental lamina, and the bud and early cap stages of tooth development in hagfish, are remarkably similar to that observed for reptilian and mammalian odontogenesis. Curiously, subsequent stages of horny tooth development more closely resemble hair and feather morphogenesis (see Maderson 1983) rather than the bell and crown stages that characterize fish, shark, frog, alligator and mouse tooth development (see discussions by Maderson 1983, Thomson 1988, Slavkin et al 1991). Moreover, hagfish horny tooth matrix forms within the enamel organ epithelium rather along the epithelial-mesenchymal interface that characterizes vertebrate tooth formations (see Slavkin et al 1991).

More recently, significant progress has been made in the elucidation of the amelogenin primary sequence for five mammalian species (see recent reviews by Fincham et al 1992, Zeichner-David et al 1995). In rodents one amelogenin gene is located on the X chromosome (Lau et al 1989) and appears to produce seven different transcripts by alternative splicing mechanisms (Lau et al 1992, Simmer et al 1994). In humans and cows one amelogenin gene is localized on the X chromosome and the other amelogenin gene is located on the Y chromosome (Lau et al 1989, Salido et al 1992, Gibson et al 1991). Using Southern hybridization and immunological criteria, an amelogenin gene and translation product has been identified in embryonic reptilian (*Alligator mississippiensis*) teeth (Slavkin et al 1984). Immunological assays demonstrated that amelogenin antigens are present in teeth of the adult frog *Rana pipiens*, but not during the tadpole stages of tooth development (Herold et al 1989).

Hagfish	YQSMIRQPY	PSYGYEPMGG	WLHHQIIPVL	SQQHPSSHNR	LPHHIIPVQ	S>
	^	^	^	^	^	^
	Mouse	YQSMIRQPY	PSYGYEPMGG	WLHHQIIPVL	SQQHPSSHNR	LPHHIIPVQ
		^	^	^	^	^
	Rat	YQSMIRQPY	PSYGYEPMGG	WLHHQIIPVL	SQQHPSSHNR	LPHHIIPVQ
		^	^	^	^	^
	Bovine	YQSMIRHPY	PSYGYEPMGG	WLHHQIIPVL	SQQHPSSHNR	LPHHIIPVQ
		^	^	^	^	^
	Human X	YQS-IRPPY	PSYGYEPMGG	WLHHQIIPVL	SQQHPSSHNR	LPHHIIPVQ
		^	^	^	^	^
	Human Y	YQSMIRPPY	SSYGYEPMGG	WLHHQIIPVL	SQQHPSSHNR	LPHHIIPVQ
		^	^	^	^	^
	Human Fetal	Y	PSYGYEPMGG	WLHHQIIPVL	SQQHPSSHNR	LPHHIIPVQ
		^	^	^	^	^

FIG. 6. Phylogenetic comparison of amelogenin gene expression during hagfish, mouse, rat, bovine and human tooth development. Sequence comparison demonstrates homology between amelogenin mRNAs of these extant vertebrates.

Herold and his colleagues suggested that the common ancestral gene for amelogenin appears in the amphibians. Several phylogenetic surveys of amelogenin-like antigens demonstrated immunolocalization within bony and cartilaginous fish, frogs, alligators and mammals (see Herold et al 1980, 1989, Slavkin et al 1982, 1983, 1984, 1991). Present evidence demonstrates that the exon 4 encoded region of the amelogenin gene is expressed in agnathan, reptilian and mammalian teeth. These lines of evidence are further supported by mRNA phenotyping suggesting homologies between agnathans and mammalian amelogenin transcripts (Slavkin & Diekwisch 1996).

In summary, we suggest that amelogenin tooth proteins are expressed in a wide variety of species throughout vertebrate evolution. Although amelogenin proteins are present invariably in all species, patterns of enamel crystal formation are featured in a heterochronic fashion in vertebrate phyla. The characteristic patterns of enamel crystal formation in mouse tooth development appear to reflect or recapitulate distinct features of early vertebrate enameloid biomineralization. We conclude that common molecular and morphological strategies during tooth development are highly conserved between all extant vertebrates.

Acknowledgements

We thank P. Bringas Jr., A. Fincham, E. Lau, S. Groff-Millar, V. Santos, J. Simmer, M. Snead and M. Zeichner-David for their support. These investigations were in part supported from research grants DE-02848, DE-09165 and DE-06425, National Institute of Dental Research, National Institutes of Health, United States Public Health Service.

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DISCUSSION

Zeichner-David: In your PCR experiments which primers did you use to amplify the amelogenin gene?

Slavkin: We obtained hagfish at various stages of tooth development from young adults as well as other non-dental tissues. The microchemical RNA extract samples were then assayed for mRNA phenotyping using 5' and 3' primers designed to include or exclude one or more of the seven exons that constitute the mammalian amelogenin gene structure.

Zeichner-David: The reason I asked this question is because in the case of the amelogenin genes, amplification is very much primer specific. For example, using DNA obtained from Hertwig's epithelial root sheath cells as a template, amplification is only observed when primers encoding exons 6 and 7 are used.

Slavkin: These studies are still in progress. Presently, two lines of evidence support our conclusion that an archetypical amelogenin gene is found within the agnathan genome and that it is expressed during tooth formation. The hagfish sequence information generated from reverse transcriptase (RT)-PCR fragments suggests homology with mouse amelogenin. The second line of evidence shows that sequence-specific polyclonal antibodies directed against the N-terminal sequence, as well as the sequence encoded within exon 4, both appeared to identify amelogenin translation products in hagfish teeth. We have not as yet completed genomic DNA comparisons nor have we completed *in situ* hybridization studies in lampreys or hagfish.

Rosenbloom: Exon 4 is one of the least conserved exons, and bovine exon 4 is practically never used, even though there's no clear reason why it shouldn't be.

Slavkin: It is definitely used in the hagfish.

Yamada: Did you find any evidence for alternative splicing in the hagfish?

Slavkin: No, there was only a single band.

Yamada: What is the role of amelogenin in hagfish development?

Slavkin: It's distributed in the cells as well as in the adjacent extracellular matrix. It's a constituent of the matrix, but we have no idea what its function might be since hagfish teeth are not mineralized, in contrast to bony fish or mammals.

Diekwisch: Hagfish teeth are very hard — they will even scratch a new diamond. We don't know the reason for this. We did not find any long crystals, although we did find that there was a relatively high calcium to phosphate ratio.

Yamada: Is it possible to do genetic manipulations of these animals?

Slavkin: The main problem is that it is extremely difficult to breed them. It may be easier to breed lampreys.

Deutsch: How large are hagfish and at what depth are they found in the sea?

Slavkin: A small hagfish is about 1 m in length, although off of the coast of Santa Barbara, California, they can reach up to 4 m in length. They are usually found at significant depths below sea level.

Deutsch: In a recent review, I read that amelogenin DNA elements have been found in fish, which is contrary to what was thought or published in the past. The authors commented, however, that they did not know whether the protein itself was expressed.

Lyngstadaas: This was our work. We did zoo blots with DNA extracted from different vertebrate species. Among them was the seawolf, *Anarrhichas lupus*, which is found in the Northern Atlantic ocean. The dentition of this fish changes once a year. It is a highly specialized dentition designed to masticate molluscs. When erupting, the teeth exhibit a surface layer that is more mineralized than the dentine. This layer is gradually worn away. When digested with the restriction enzyme *EcoRI*, an 18 kb fragment from *A. lupus* DNA hybridized strongly with the mouse amelogenin cDNA under stringent conditions. This suggests that this fish has a genomic DNA sequence similar to parts of the murine amelogenin gene (Lyngstadaas et al 1990). So far, we have not analysed the teeth for amelogenin proteins, so we do not know whether these proteins are actually present in this animal.

Slavkin: The new advances have enabled us to gather an increasingly complete picture of the phylogenetic distribution of enamel gene products. I recall that Richard Herold used amelogenin antibodies to localize antigens in the enameloid of lower vertebrates (Herold et al 1980). More recently, Herold and co-workers (Herold et al 1989) showed the less than anticipated appearance of enamelines but not amelogenins in tadpole frog teeth, whereas both enamelines and amelogenins were expressed in the adult teeth of *Rana pipens*.

Diekwisch: We have also detected amelogenin protein in sharks, rays, guppies, the Australian lungfish, axolotl and frogs.

Snead: Sometime ago you proposed that prismatic enamel, versus aprismatic enamel, depended on the occurrence of amelogenins. This makes the hagfish an iconoclast in this respect.

Slavkin: Yes, that is correct.

Sharpe: I would like to clarify, for the benefit of non-molecular biologists, that although there are HOX genes that are expressed at the level of rhombomere 2, those genes are not expressed in the neural crest cells that migrate out from rhombomere 2. Therefore, to my knowledge, HOX genes are not expressed in the oral epithelium or in the neural crest-derived mesenchyme of the first branchial arch, so they do not have a direct role in odontogenesis.

Slavkin: HOX genes are all restricted to the anteroposterior gradient of an organism, whether it be *Amphioxus*, *Drosophila* or the mouse. However, homeotic-like genes are expressed in the first branchial arch, suggesting that these close relatives are involved with dorsoventral rather than anteroposterior patterning.

Sharpe: There are homeodomain-containing genes that are not part of the HOX gene clusters. Homeobox genes, such as the *Dlx* and *Msx* families, are related to the *Drosophila* homeotic genes because they contain the homeodomain, but they are not evolutionarily direct relatives of the *Drosophila* homeotic genes.

Slavkin: Andrew Lumsden and his colleagues showed that *Msx1* and *Bmp4* are expressed within the neural ectoderm of the hindbrain, and that *Msx1* expression reappears in the first branchial arch (Graham et al 1994).

Sharpe: *Msx1* and *Msx2* are expressed in many neural crest derivatives, and it's clear that *Msx1* is required for normal tooth development (this is probably also true for *Msx2* as well). A third member of that family, *Msx3*, is probably the archetypal gene. Peter Holland has shown that *Amphioxus* has a single MSX gene, which is expressed predominantly in the nervous system. *Msx3* is only expressed in the nervous system, and not in any of the areas that express *Msx1* and *Msx2*, such as neural crest and teeth.

Slavkin: Have you looked for MSX genes in the lamprey?

Sharpe: No. It would be interesting to determine the point at which the single MSX gene duplicated. We assume that this occurred at the time when the true vertebrates evolved because the main difference between their expression patterns is in the neural crest.

Snead: Do *Amphioxus* and hagfish determine sex by allelic exclusion or do they have sex-determining factors?

Sharpe: I don't think this is known.

Nanci: We have been looking at the regeneration of scales in a primitive actinopterygian fish (*Calamoichthys calabaricus*). We have observed the deposition of a matrix that looks like the aprismatic enamel layer of mammalian teeth in the regenerating scales of this fish. There is also a basement membrane-like structure at the interface between the epithelial cells and the ganoin layer. We have shown that several anti-amelogenin antibodies recognize material in the ganoin layer of the scales, suggesting, if not demonstrating, that amelogenin is present there and that it is, evolutionarily, an early product.

Mann: I would like to remind everyone that many invertebrates have teeth. Some years ago we looked at limpet teeth, which have a base and a cusp (Mann et al 1986). The base is granular and is impregnated with calcium phosphate. The cusp is made of iron oxide and it is infiltrated with silica. The iron oxide crystals are relatively long and are interwoven in a similar general structure as the scanning electron micrographs that Alan Boyde presented (Boyde 1997, this volume). Very little, if anything, is known about the biochemistry of this system. There is an epithelial layer over the teeth, and another interesting aspect is that the teeth are on a sort of conveyor belt, so that mineralization occurs in a temporal and spatial sequence.

Veis: Over 10 years ago we published that sea urchin teeth contain some acidic matrix proteins which can be immunoprecipitated with anti-phosphophoryn antibodies (Veis et al 1986).

Simmer: It appears that the bottom has fallen out of the evolution of amelogenins. I would like to set a limit in that we have checked and determined that bacteria do not express amelogenins (J. P. Simmer, unpublished results 1994)!

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The protein composition of normal and developmentally defective enamel

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Abstract. The development of human enamel involves a complex series of events including the secretion and degradation of a unique extracellular matrix. Ameloblasts progress through a succession of cellular phenotypes executing specialized secretory and regulatory functions. When performing optimally, ameloblasts produce a highly structured and mineralized tissue. Given the elaborate developmental events required for normal enamel formation, it is not surprising that a variety of enamel malformations arise from defects in matrix synthesis, secretion and extracellular processing. Normal matrix secretion and post-secretory processing by ameloblasts can be affected by a variety of hereditary and environmental conditions. These disturbances can result in an abnormal amount and/or composition of matrix proteins, and subsequently, an altered enamel structure and/or mineral content. For example, abnormal matrix removal during enamel maturation apparently contributes to hypomineralization associated with dental fluorosis. Incomplete matrix removal can also occur in several different forms of the hereditary condition amelogenesis imperfecta. Specific types of this condition can have retention of substantial enamel protein (e.g. 5% by weight) that is, at least in part, composed of amelogenin and/or its breakdown products. Characterization of the enamel proteins in teeth affected by developmental disturbances can provide insight into the pathogenesis and normal formation of this highly specialized tissue.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 85-106

Enamel formation involves a remarkable orchestration of complex molecular and cellular events that culminate in one of the most uniquely structured tissues and the most highly mineralized tissue in the body. Ameloblasts respond to as yet undefined signals/transduction messages that prompt them to produce and secrete numerous extracellular substances in a highly controlled temporal-spatial fashion. Although amelogenin is the most abundant extracellular ameloblast product, a variety of diverse and essential materials are produced. The enamel matrix purportedly regulates mineralization, controls the morphology of the crystallites and may act as a mineral nucleator (Fincham et al 1995). Although the specific functions of the enamel matrix proteins remain to be defined, there is no question that altered synthesis,

secretion and extracellular processing can result in enamel that is defective in amount, structure and composition.

The ameloblasts proceed through a life cycle allowing them to secrete and remove a specialized matrix and to regulate the ionic environment so that mineralization can proceed. Shortly after differentiation, the ameloblasts begin to express tuftelin, a unique acidic protein with a molecular weight of about 46 kDa (Deutsch et al 1991). The predominant matrix protein in secretory enamel (about 90%) is amelogenin, which has a molecular weight of about 26 kDa (Fincham et al 1992). The term enamelin has been applied to a group of enamel proteins with molecular weights ranging from 32 to 142 kDa (Fukae et al 1993). The enamelines are described as sialylated glycoproteins and are present in relatively small amounts (Simmer & Fincham 1995). Other matrix components include short-lived sulfated proteins and proteinases.

Proteinases are active in developing enamel and apparently function in the process of selective protein degradation and removal (DenBesten & Heffernan 1989, Smith et al 1989, Tanabe et al 1992). Both serine proteinases and metalloproteinases have been identified in developing enamel. Proteinases are produced by ameloblasts early in enamel development, with maximal activity appearing during the transition and maturation developmental stages (Robinson et al 1990). Several specific amelogenin cleavage sites have been identified, including one that produces the N-terminal tyrosine-rich amelogenin protein (TRAP) (Sasaki et al 1991) and also the first proline on the C-terminus (Moradian-Oldak et al 1994). Controlled removal of enamel proteins may provide the mechanism for regulating the rate of crystal growth as well as defining crystallite morphology (Robinson et al 1990, Fincham et al 1991, Brookes et al 1995). Investigators hypothesize that inhibition of proteolysis leads to retention of protein, thereby interfering with the maturation process and crystal growth. This may be the aetiology of several enamel pathologies, including certain forms of amelogenesis imperfecta and dental fluorosis (DenBesten & Giambro 1995, Wright 1995).

Although enamel begins as a protein matrix, it becomes a mineralized tissue that is approximately 85% mineral by volume and 96% by weight. The protein content of enamel changes dramatically during normal development ranging from about 20% protein by weight during the secretory stage to 7% at the beginning of the maturation stages (Deutsch & Alayoff 1987). Ultimately, the ameloblasts remove virtually all of the original matrix as mineralization progresses. Fully developed normal human enamel contains 0.01–1% protein by weight (Robinson et al 1975, Deutsch & Alayoff 1987). During development, the amino acid composition of the enamel matrix changes as amelogenin is degraded and lost (Fincham et al 1982). Newly secreted amelogenin-rich enamel matrix has a substantial proline content (25–30% of the total number of residues), whereas mature enamel has a reduced proline content and elevated glycine and glutamic acid composition. In certain pathological conditions the protein content and composition of mature enamel can be markedly altered.

There are over 100 aetiologies of abnormal enamel formation (Small & Murray 1978). Environmental influences and/or genetic alterations can change virtually any

developmental phase or specific process, thereby resulting in aberrant enamel formation. Abnormal enamel matrix secretion and extracellular processing are implicated in many of these developmental disturbances. Although premature cessation of ameloblast function can result in thin or pitted enamel, the processes involved in the development of hypomineralized enamel are less clear and apparently complex. There is growing evidence that a variety of diverse mechanisms can result in the alteration of the protein content of fully developed enamel.

It is now known that mutations in the amelogenin gene can result in both hypoplastic and hypomineralized enamel (Lagerström et al 1991, Lench & Winter 1995). Although the molecular defects are being identified for several conditions having enamel malformations, the actual developmental mechanisms resulting in enamel defects are unknown. The association of mutations in the amelogenin gene with enamel defects indicates an important role for this extracellular protein in enamel formation. Several unique enamel products have recently been proposed as possible aetiologies for autosomally inherited enamel defects (Deutsch et al 1995). Other enamel defects are thought to result from abnormal, incomplete or delayed removal of amelogenin or other enamel extracellular matrices (Wright & Butler 1989). Thus, the proteinases active during enamel development are also likely aetiological candidates for at least some enamel malformations.

It has been suggested that under certain pathological conditions extraneous materials or non-ameloblast-derived proteins could contaminate the growing enamel crystals, thereby inhibiting normal mineralization (Robinson et al 1992). Although little is known about the organic composition of enamel in pathological conditions, we have begun to characterize human enamel from a variety of disorders. Therefore, the purpose of this chapter is to present our initial characterization of the enamel proteins in a variety of environmentally induced and hereditary conditions that are associated with enamel defects.

Materials and methods

We collected exfoliated or therapeutically extracted primary and permanent teeth from individuals for analysis, and categorized them based on careful clinical and historical assessment of the donors. We analysed moderately fluorosed teeth that had developed in an area with 3.2 ppm fluoride in the drinking water and teeth with the major forms of amelogenesis imperfecta, using normal teeth of the same type as controls. Having cut thin sections from fully developed teeth using a low speed saw with H₂O lubrication, we dissected enamel samples for amino acid analysis, and PAGE and Western blot analyses. For the amino acid analysis, we weighed the samples, hydrolysed them in 7M HCl under vacuum after flushing with N₂ and analysed them by ninhydrin derivatization using an HPLC system fitted with a cation-exchange column (Yamauchi et al 1986). We estimated the total enamel protein content from the amino acid content, and monitored dentine contamination by the presence of

hydroxyproline, which is not normally present in enamel but is one of the major amino acids in the dentine matrix.

Although samples of enamel with amelogenesis imperfecta could be finely ground directly into SDS buffer for gel electrophoresis and Western blot analyses, we had to dissect and demineralize normal and fluorosed enamel samples (approximately 60 mg) in 3500 MW cut-off dialysis bags using 10% acetic acid so that they could be lyophilized prior to electrophoresis. For a positive control for all amelogenin Western blot analyses, we harvested and ground rat secretory enamel from freshly extracted teeth directly into sample buffer. We ran the samples on 5–20% gradient SDS-PAGE gels using standard conditions (Laemmli 1970).

For Western blotting, we loaded a sample volume of each specimen similar to that used for the SDS-PAGE analysis into additional lanes of the gradient gel prior to electrophoresis. Following electrophoretic separation of the enamel proteins, we transferred proteins on half of the gel to nitrocellulose paper using standard techniques. We stained the other half of the gel with Coomassie Blue and silver stain. After blocking the nitrocellulose overnight with a 1% powdered milk solution, we exposed the blots overnight at 4 °C to a polyclonal anti-amelogenin antibody (1:1400 dilution) raised in rabbits against recombinant mouse amelogenin protein (Simmer et al 1994) (courtesy of M. Zeichner-David). We visualized cross-reactivity using an alkaline phosphatase-conjugated second antibody.

Enamel proteins in fluorotic teeth

Environmental disturbances of enamel formation are extremely diverse. Environmental influences can have a direct effect on the ameloblasts, altering their secretory function or viability. Environmental factors could also influence enamel formation by altering processes such as crystal growth, crystallite annealing or matrix processing. For example, ingestion of excess amounts of various elements or compounds can act directly on the ameloblasts and/or the developing matrix, depending on the dose and material. Dental fluorosis is probably the most studied and, at present, arguably the most important toxicity issue regarding enamel formation.

Dental fluorosis results from ingestion of excess amounts of fluoride, producing enamel hypomineralization in a dose-response fashion. Fluorosed enamel is characterized by various degrees of hypomineralization that apparently result from a variety of mechanisms, including abnormal post-secretory enamel matrix processing (DenBesten & Giambro 1995). Animal studies show that increased consumption of fluoride leads to abnormal maturation of the enamel and delayed removal of the extracellular matrices (DenBesten & Crenshaw 1987). It has been suggested that elevated fluoride concentrations could inhibit proteolysis, thereby causing either incomplete or delayed removal of the extracellular matrices.

Several investigators have described fluorosed enamel as having an increased organic content based on histological and histochemical methodologies (DenBesten

& Giambro 1995). Eastoe & Fejerskov (1984) reported that mature fluorosed human enamel had a similar total protein content to normal enamel, but demonstrated a higher proportion of immature matrix proteins known as amelogenins (rich in proline, glutamic acid, aspartic acid and histidine).

Our studies showed that the protein content of fluorosed enamel from nine teeth was elevated compared with control enamel (the mean total protein in fluorosed enamel was 0.27% [S.D. = 0.183], whereas it was 0.11% [S.D. = 0.026] in control enamel). The normal enamel protein content ranged from 0.07% to 0.14%, whereas the fluorosed enamel protein content ranged from 0.03% to 0.56%. The amino acid compositions for control and fluorosed enamel were similar and are pictorially presented as rose diagrams in Fig. 1. Both fluorosed and normal enamel were high in glycine, glutamic acid and leucine. The enamel amino acid compositions were similar for all the fluorosed and normal teeth with most amino acids showing little sample-to-sample variation.

This study of moderately fluorosed human enamel demonstrated that the total protein content of fluorosed enamel was similar to that reported by Eastoe & Fejerskov (1984). Although fluorosed enamel had a significantly greater protein content compared with the normal enamel ($P = 0.0001$, unpaired Student's *t* test), the protein contents of both the fluorosed and normal enamel were within the ranges reported as normal (Robinson et al 1975, Deutsch & Alayoff 1987).

Gel electrophoresis, under reduced or non-reduced conditions, showed generally similar banding patterns for fluorosed and normal enamel. Protein bands could be seen at 200, 50, 30 kDa and below in both the control and fluorosed enamel samples. There were some distinct bands in the fluorosed enamel, whereas the control enamel produced principally a broad diffuse pattern (Fig. 1). Analysis of the enamel protein cross-reactivity with anti-amelogenin antibody showed no reaction in either the normal or fluorosed enamel samples in the low molecular weight areas expected for amelogenin. This indicates that if amelogenin is retained, it is present in amounts below our detection limits.

Our analysis of the fluorosed enamel did not reveal an amelogenin-like amino acid profile, as was shown in a study by Eastoe & Fejerskov (1984) nor cross-reactivity to an anti-amelogenin antibody. These results do not support the hypothesis of permanently retained amelogenin or amelogenin fragments in fully developed fluorotic enamel. Although the protein content of fluorotic enamel had an amino acid composition similar to normal enamel, the fluorosed enamel had more protein and a variety of molecular weight species not seen in normal enamel. This indicates that exposure to excess fluoride may indeed have some effect on the matrix during tooth development.

In order to explain the complex structural and compositional features of fluorotic human enamel it seems probable that several diverse mechanisms are involved (Bawden et al 1995, DenBesten & Giambro 1995). Although we have provided evidence that fluorotic human enamel does not demonstrate permanent amelogenin retention, there are several studies indicating a delayed removal of amelogenin during enamel maturation that could affect crystallite growth at a critical period

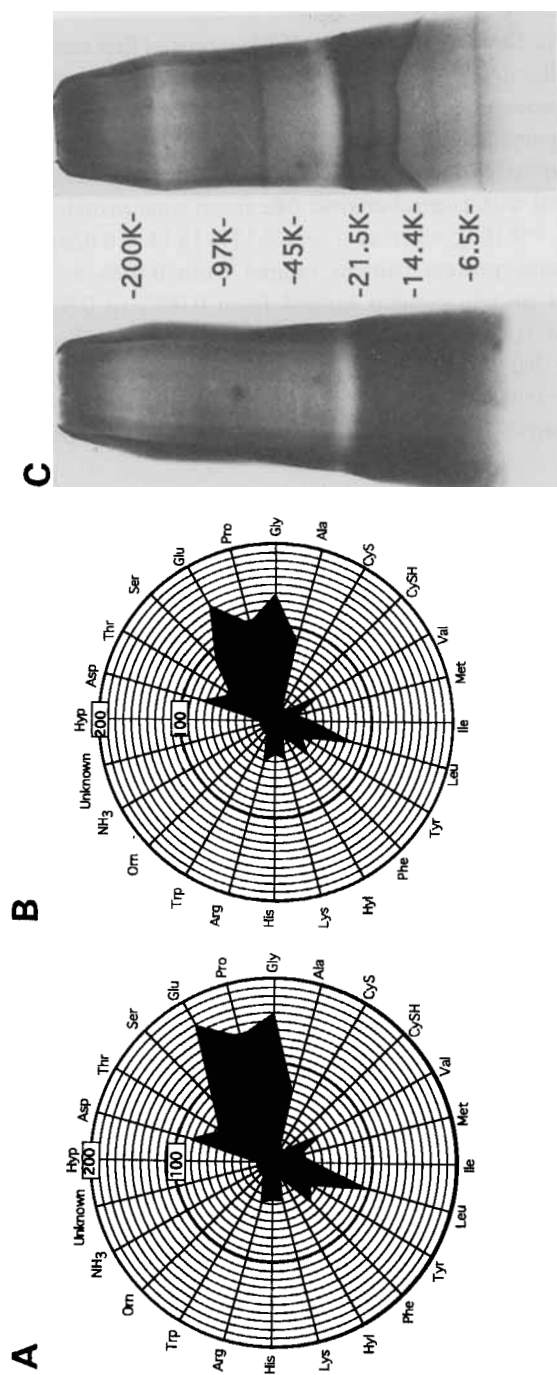


FIG. 1. The amino acid compositions of (A) normal permanent human enamel (0.11% mean protein, $n = 10$) and (B) moderately fluorosed permanent human enamel (0.27% mean protein, $n = 9$) are similar. (C) SDS-PAGE analysis (silver stain) shows that fluorosed enamel (right) shows several distinct molecular weight proteins that are not observed in normal enamel (left).

(DenBesten & Giambro 1995). Our investigation indicates that the majority of amelogenin matrix is ultimately removed. Exposure to excess fluoride during enamel development appears to produce tissue changes through interactions with the forming mineral, the immature enamel matrix and the ameloblasts. Although this investigation indicates that there may be both quantitative and qualitative differences in the organic matrix of fluorosed enamel compared with normal enamel, further studies are required to determine if these differences are primarily involved in abnormal enamel development or if they occur secondarily as a result of fluorosed enamel hypomineralization.

Enamel proteins in amelogenesis imperfecta

The condition amelogenesis imperfecta comprises a group of hereditary disorders characterized by alteration of the quantity and/or quality of enamel in humans, and it is frequently associated with significant oral morbidity (Wright 1995). In its mildest form amelogenesis imperfecta displays discolouration and abnormal morphology of the clinical dental crowns, and in its most severe presentation the enamel is hypomineralized causing it to be abraded from the teeth shortly after emergence into the oral cavity (Wright et al 1995). The three main types of amelogenesis imperfecta and their presumed developmental defects are: hypoplastic amelogenesis imperfecta, i.e. thin or pitted enamel due to appositional defects; hypocalcified amelogenesis imperfecta, i.e. hypomineralization of crystallites due to defective crystallite nucleation and subsequent mineralization; and hypomaturational amelogenesis imperfecta, i.e. defects in crystallite growth during the maturation phase of enamel development (Witkop & Sauk 1976). The diverse clinical presentations of amelogenesis imperfecta are thought to result from specific gene defects involving the deposition, calcification and maturation of enamel.

Protein determination based on the amino acid analyses showed that there were marked differences in the quantity of enamel protein in different types of amelogenesis imperfecta teeth ($n = 12$ individuals with amelogenesis imperfecta/30 amelogenesis imperfecta teeth) compared with normal enamel (Table 1). Although there was considerable variation, most of the amelogenesis imperfecta teeth had substantially more protein per weight enamel than the control enamel. The hypoplastic amelogenesis imperfecta types had the lowest enamel protein levels, which varied from being similar to that of normal enamel to being mildly increased. Two hypoplastic amelogenesis imperfecta cases had protein contents similar to normal enamel. One of these was a heterozygous female with X-linked amelogenesis imperfecta and the other was a female with rough hypoplastic amelogenesis imperfecta. In contrast, both hypocalcified and hypomaturational amelogenesis imperfecta enamels had markedly elevated protein levels compared with normal enamel. The greatest increases in enamel protein content were seen in cases of hypomaturational amelogenesis imperfecta (4.4–6.8%). Fully developed hypomaturational amelogenesis imperfecta enamel showed an approximately 20- to 30-fold increase in the protein content compared with normal enamel.

TABLE 1 Protein content of normal and amelogenesis imperfecta enamel

<i>Group/case</i>	<i>n</i>	<i>Inheritance</i>	<i>Mean protein (%)</i>	<i>Range</i>
Hypoplastic	1	X-linked/AD ^a	0.2	NA
	1	X-linked	0.4	NA
	1	sporadic	1.9	NA
	1	AD	2.2	NA
Hypomaturation	5	AR	5.0	1.6–7.3
	2	AR	6.8	6.3–7.3
	4	AR	4.4	3.8–4.7
Hypocalcified	4	AD	4.8	3.8–5.8
	4	AD	3.2	1.4–4.5
	3	AD	4.4	2.3–5.6
	2	AD	0.04	0.01–0.07
	2	AD	4.4	4.3–4.7
Control permanent	21	NA	0.15	0.06–0.75
Control primary	7	NA	0.22	0.06–0.45

^aNot certain.

AD, autosomal dominant; AR, autosomal recessive; NA, not applicable.

Examination of the enamel amino acid compositions of the different types of amelogenesis imperfecta showed marked differences between the major types and similarities between most cases of the same type. The mean amino acid compositions for normal enamel and the different amelogenesis imperfecta types are presented in Figs 2–5. Two hypoplastic amelogenesis imperfecta cases exhibited an elevated glycine content (Fig. 3A) and two hypoplastic amelogenesis imperfecta cases demonstrated a slightly increased proline content (Fig. 3B), although the overall amino acid compositions in these cases were similar to normal enamel. One hypoplastic amelogenesis imperfecta case displayed an amelogenin-like amino acid composition rich in proline, glutamic acid, tyrosine and leucine (Fig. 3C).

The hypocalcified amelogenesis imperfecta enamel amino acid profiles were remarkably similar to each other and appear different from the other types of amelogenesis imperfecta (Fig. 4). Hypocalcified amelogenesis imperfecta enamel showed an increased tyrosine and leucine content compared with normal enamel. None of the hypocalcified amelogenesis imperfecta cases had an elevated proline content. Hypomaturation amelogenesis imperfecta enamel proteins were rich in proline, tyrosine and histidine, giving them an amelogenin-like profile (Fig. 5). Although there was some variation between the three cases of hypomaturation

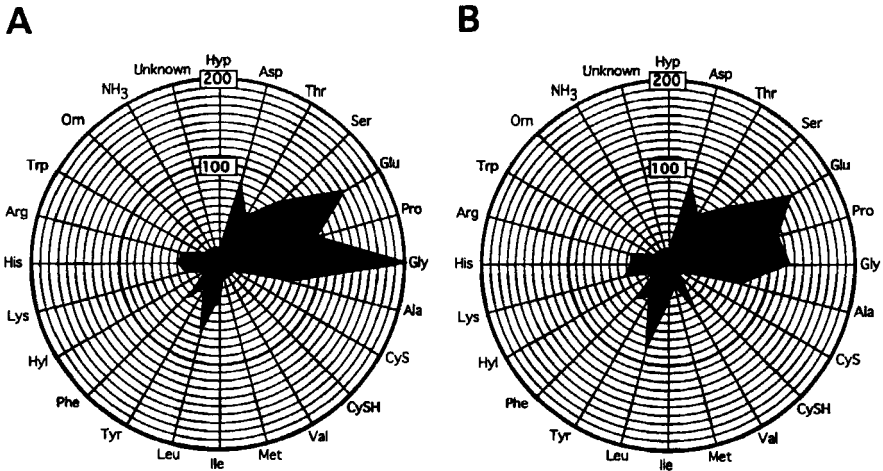


FIG. 2. The amino acid compositions of (A) normal primary enamel (0.22% mean protein, $n=7$) and (B) normal permanent enamel (0.15% mean protein, $n=21$) are similar. Primary enamel has an elevated glycine content compared with permanent enamel.

amelogenesis imperfecta studied, both the protein quantity and mean amino acid profiles were similar.

Analysis of the enamel proteins using SDS-PAGE and Western blotting revealed marked differences between the various amelogenesis imperfecta ($n=7$) and control enamel samples. Normal enamel that was demineralized created variable gel patterns, with some samples producing no distinct banding and only broad smears down the entire gel lane. In other normal enamel samples discrete bands at about 70 kDa, 60 kDa and occasionally between 12 kDa and 30 kDa were seen. None of the amelogenesis imperfecta enamel samples produced banding patterns similar to those observed in normal enamel. Electrophoretic patterns were somewhat variable even between similar amelogenesis imperfecta types. Furthermore, none of the normal enamel samples showed cross-reactivity with the anti-amelogenin antibody (results not shown).

We analysed two hypoplastic amelogenesis imperfecta cases by SDS-PAGE. The hypoplastic amelogenesis imperfecta case that had an amelogenin-like amino acid composition produced a smeared gel lane pattern with a discernible band at about 18 kDa. Western blot analysis of this case showed cross-reactivity of the enamel proteins to the anti-amelogenin antibody (results not shown).

All of the hypocalcified amelogenesis imperfecta cases showed a diverse composition of enamel protein species ranging from 6 kDa to 200 kDa (Fig. 4). Interestingly, Western blot analysis using the anti-amelogenin antibody revealed that hypocalcified amelogenesis imperfecta enamel showed multiple cross-reactive bands of

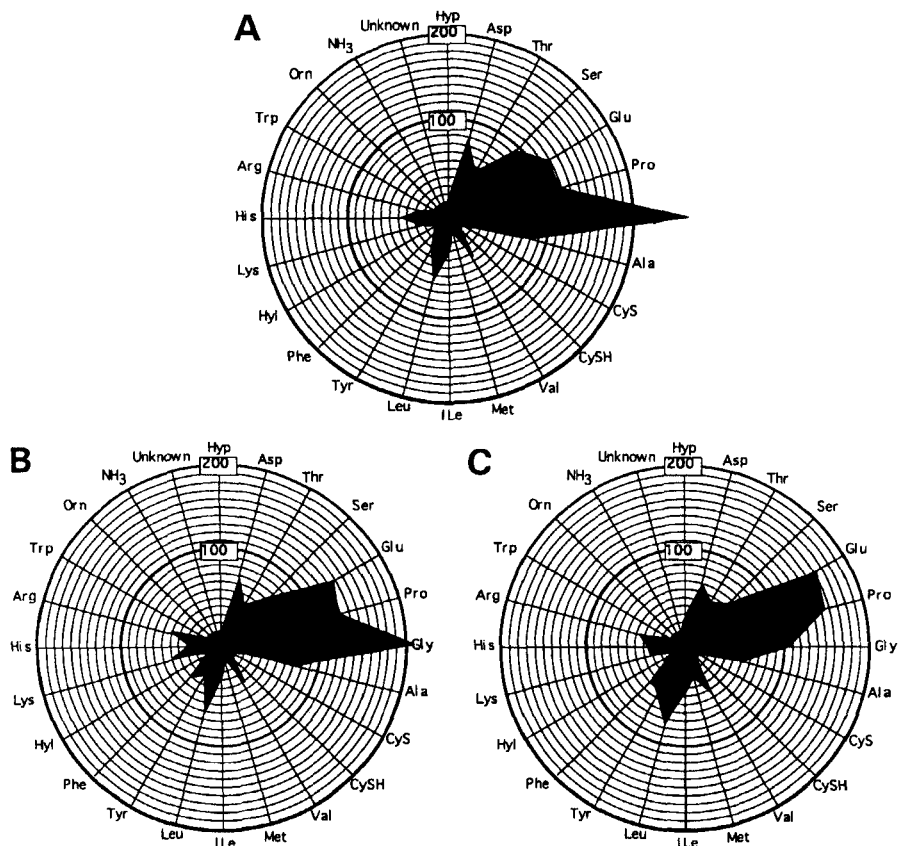


FIG. 3. The diverse amino acid compositions in hypoplastic amelogenesis imperfecta enamel. (A) Mean of two cases (both probably X-linked) that have a protein content similar to normal enamel (0.3% mean protein), with the exception of a marked elevation in glycine. (B) Mean of two cases showing a slightly elevated proline and glycine content compared with normal enamel (1.4% mean protein). (C) One hypoplastic amelogenesis imperfecta case with an amelogenin-like amino acid composition (2.2% protein).

variable molecular mass. In some cases material ranging from 12 kDa to about 200 kDa cross-reacted with the anti-amelogenin antibody, whereas in other samples only specific low molecular weight material reacted. All hypocalcified amelogenesis imperfecta cases tested ($n=3$) showed material that cross-reacted with the anti-amelogenin antibody.

Hypomaturation, hypocalcified and X-linked hypoplastic amelogenesis imperfecta samples all produced a band at about 14 500 Da that was not seen in the normal enamel samples, indicating the possible retention of amelogenin. Hypomaturation amelogenesis

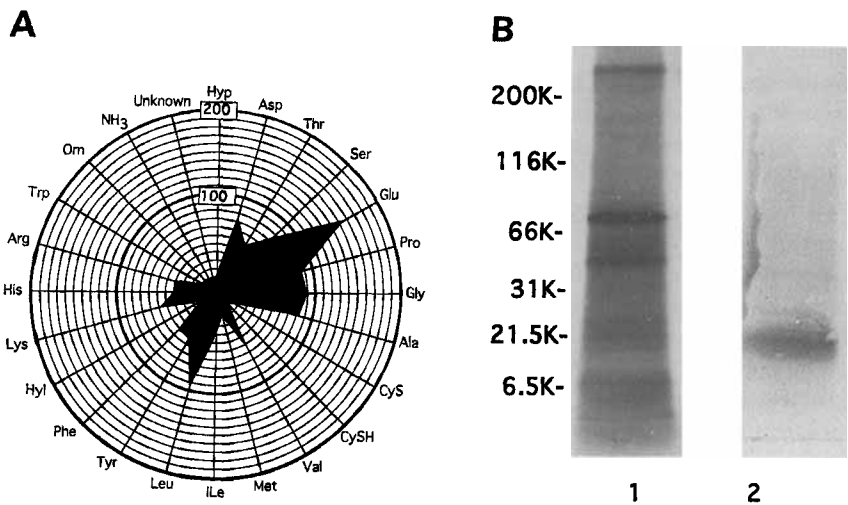


FIG. 4. (A) The mean amino acid composition of five unrelated hypocalcified amelogenesis imperfecta cases (3.7% mean protein, $n = 14$ teeth) is similar to normal enamel. (B) SDS-PAGE analysis shows that a variety of molecular weight proteins are present in the enamel (lane 1), and several of the low molecular weight species show cross-reactivity to the anti-amelogenin antibody (lane 2).

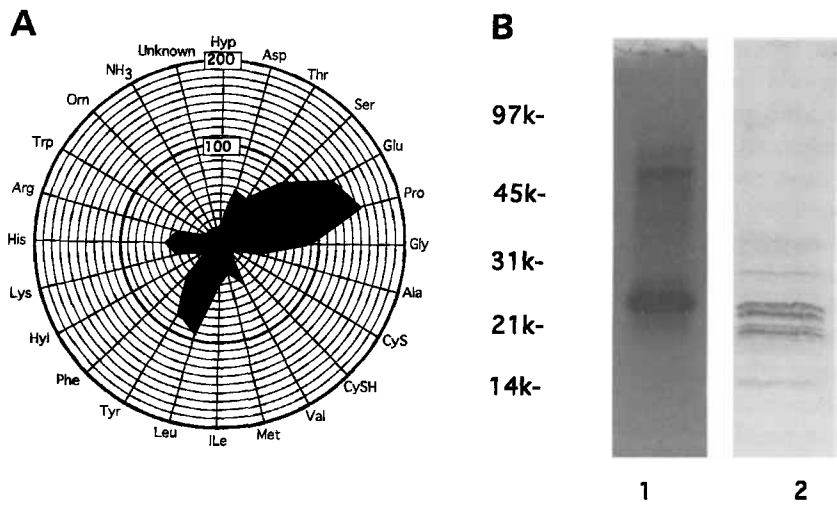


FIG. 5. (A) The amelogenin-like mean amino acid composition of three cases of hypomaturational amelogenesis imperfecta enamel, which have a high proline, tyrosine and histidine content compared with normal enamel (5.4% mean protein). (B) A preponderance of low molecular weight enamel proteins are identified by SDS-PAGE analysis (lane 1), several of which cross-react with the anti-amelogenin antibody (lane 2).

imperfecta enamel typically showed a predominant composition of low molecular weight species (less than 40 kDa) (Fig. 5). One case of hypomaturational amelogenesis imperfecta demonstrated a pronounced band at about 50 kDa that was not present in the other hypomaturational amelogenesis imperfecta cases. All cases of hypomaturational amelogenesis imperfecta enamel ($n = 3$) showed anti-amelogenin antibody labelling. In two cases cross reactivity of the anti-amelogenin antibody was primarily limited to material less than 30 kDa (Fig. 5). Hypomaturational amelogenesis imperfecta enamel contained proteins of a diverse molecular mass, with multiple molecular weight materials from 18 kDa to 200 kDa showing cross-reactivity to the anti-amelogenin antibody.

The analysis of different types of amelogenesis imperfecta enamel indicates that the proteins differ both quantitatively and qualitatively between the various disorders. Characterization of these amelogenesis imperfecta cases supports earlier observations that hypocalcified and hypomaturational amelogenesis imperfecta types have an increased enamel protein content compared with other types of amelogenesis imperfecta or normal enamel (Wright et al 1992). Furthermore, individuals affected with hypomaturational and hypocalcified amelogenesis imperfecta appear to have enamel proteins that are relatively consistent in amino acid composition between cases with the same diagnosis. In contrast, there appears to be substantial variation in the hypoplastic amelogenesis imperfecta cases with regards to both the quantity and composition of enamel proteins. The marked alteration and diversity of enamel proteins in the different amelogenesis imperfecta types supports the hypothesis that different developmental mechanisms are involved. The variability of inheritance patterns, phenotypes and enamel protein compositions in the hypoplastic amelogenesis imperfecta group suggests there are multiple pathological mechanisms responsible for this one group of enamel defects.

The diverse clinical manifestations, enamel protein species, ultrastructural features and different inheritance patterns indicate that the amelogenesis imperfecta disorders result from several different basic mechanisms (Bäckman 1988, Aldred et al 1992, Forsman et al 1994). Even similarities such as the retention of amelogenin or amelogenin fragments could occur due to distinctly different defects that disrupt enamel formation at different points along the complex developmental pathways. This investigation shows that all three major types of amelogenesis imperfecta can have retention of amelogenin. While direct defects of the post-secretory processing of the enamel extracellular matrices appear likely in some amelogenesis imperfecta types, retention of extracellular matrix material could also result from the synthesis of structurally altered proteins, or alterations in the timing of the developmental sequences of synthesis, secretion and degradation.

Summary

Investigation of the enamel in teeth affected with different pathological conditions reveals that there is extreme diversity in the enamel protein content. This finding is

consistent with our understanding that the final protein content of enamel is determined by multiple secretory and degradation events and that these events can be altered at a variety of developmental stages. Changes in the formation or removal of the enamel matrix appear to have dramatic consequences on the enamel structure and final composition. Investigation of fluorotic and amelogenesis imperfecta enamel indicates that both the mineral and organic components can be affected, with marked variations in severities.

Although it has been shown that an increased enamel protein content is associated with a decreased enamel mineral content (Wright et al 1995), the relationship between retention of specific proteins and crystallite defects remains to be defined. Although molecular approaches are able to define the mutations responsible for the hereditary enamel disorders, further protein chemistry and ultrastructural analyses are required to elucidate the protein-protein interactions, post-translational modifications, mineral-protein interactions and relationships in both normal and pathological enamel development.

Acknowledgement

This study was supported in part by National Institutes of Dental Research grants RO3-DE10025 and RO1-DE10489.

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DISCUSSION

Boyde: Are the teeth discoloured when they erupt, and what is the nature of the discolouration?

Wright: I've extracted and sectioned impacted, unerupted third molars and found that they're uniformly discoloured throughout. However, the colour can change after eruption due to the enamel porosity.

Yamada: We have recently mapped the ameloblastin gene to mouse chromosome 5, and the equivalent human location is chromosome 4, which is close to the autosomal amelogenesis imperfecta locus. Mary MacDougall and Birgitta Bäckman have tried to establish a linkage between the ameloblastin gene and amelogenesis imperfecta.

Slavkin: What's the prevalence of amelogenesis imperfecta?

Wright: It depends on where you look. In the USA the prevalence is 1 : 15 000 and in Israel it is 1 : 8000.

Brook: Sundell (1986) quoted a figure of 1 : 4000 in the West Coast of Sweden.

Slavkin: The majority of dental schools in North America do not include genetics in the curriculum, so it is possible that there's a disparity in these results due to under-reporting.

Bäckman: Yes, that's definitely the case. We may not diagnose all the cases of amelogenesis imperfecta because the clinical variation is so large and we may not always recognize the phenotype.

Winter: Even the American figures are doubtful because Witkop (1957) reported originally that the prevalence was 1 : 14 000 to 1 : 16 000, but subsequently he stated that the prevalence of the snow-capped teeth subtype was about 1 : 2000 (Witkop & Sauk 1976). I pointed out to him that he could not have 1 : 2000 as part of 1 : 14 000, but he never retracted his original figures.

Brook: The prevalences must vary tremendously if there are a number of people diagnosing the cases, unless careful calibrations are made. Also, when the ratios are in the order of one in so many thousand and you don't have a hundred thousand people to screen, the ratios would be distorted if there was a particular gene pool within your sample.

Snead: The United States Department of Defense has proposed phenotyping all the army inductees for post-mortem identification should the need arise. There's been a large debate about the use of this material, but the argument from the biomedical

community is that if the samples cannot be traced back then they should be made available for reverse transcriptase PCR analysis. This would provide enough samples to perform reliable mapping experiments. One could use sets of primers that flank mutations in the amelogenin gene to phenotype each individual. This would be a way of determining the genotypic incidence without knowing the phenotype.

Wright: It would determine the incidence of those specific mutations but it would not tell us anything about the remainder of the amelogenesis imperfecta types, which are the majority. For example, in my sample the majority of cases are not X-linked.

Slavkin: In the USA there are four million live births per year, so there are about 52 million children of 17 years of age and younger, and Europe is probably comparable in both birth rate and the number of children aged less than 17 years. Therefore, it would seem that there is a large demand for a new classification. We need a thorough genotyping of a large number of samples to sort this problem out. It should be an international approach, since the prevalences indicate that obtaining a suitable population is too large a problem to be solved by a single country. The high through-put genotyping technology exists, but we need to establish an international collaboration.

Rosenbloom: You showed a gel of hypomaturational amelogenesis imperfecta enamel that had been stained with Coomassie Blue and probed with the anti-amelogenin antibody. Most of the protein that reacted with the antibody did not run to the same position as amelogenin, even though the amino acid composition seemed to be very much like amelogenin. Can you comment on this?

Wright: There are many proteins that have an amelogenin-like proline profile, but are not amelogenin. We sampled enamel from the tooth, which is porous, so we may have ended up with proline-rich salivary proteins. We have performed gel and blot analyses for many amelogenesis imperfecta teeth, and found that each case was slightly different. In some amelogenesis imperfecta teeth we find high molecular weight enamel proteins that cross-react with the antibody, which could represent amelogenin aggregates.

Zeichner-David: It is possible that you are detecting ameloblastin because your gels are remarkably similar to the ones that both Yoshi Yamada's group and our group have produced using the ameloblastin antibody. These gels show a band at about 50–55 kDa and another band at a lower molecular mass. Neither of these bands cross-react with the anti-amelogenin antibody. Unfortunately, we currently know very little about ameloblastin, and we don't know whether it is retained in normal teeth or not. However, we know it is rich in proline.

Wright: We've excised some of those bands, performed amino acid analyses and tried to do some sequencing, and this has demonstrated that at least some of those higher molecular weight bands do not have an amelogenin-like composition.

Deutsch: I have a comment and a question. First, X-linked amelogenesis imperfecta represents only a small percentage of the total number of amelogenesis imperfecta cases—most are autosomally linked. Second, have you looked at the unerupted enamel of amelogenesis imperfecta teeth? Because in some of the amelogenesis

imperfecta teeth some enamel is removed by mastication forces after they erupt into the mouth (e.g. in cases of hypocalcification amelogenesis imperfecta) and in other cases, where erupted enamel is very porous, it could perhaps take up extraneous proteins such as saliva proteins, and this would therefore affect the results.

Wright: We have only looked at unerupted teeth in two cases, both of which were impacted third molars of cases of hypomaturational amelogenesis imperfecta. There were no differences in the protein compositions between erupted and unerupted teeth from the same cases. Therefore, it is unlikely that extraneous material is affecting the results, although this is always a concern.

Winter: Your results will also vary depending on which cases and on which teeth you're investigating. This is particularly relevant in the hypocalcified cases because there is phenotypic variation in different teeth. For example, some of the teeth may have extremely soft enamel and others may not be affected to the same extent.

Wright: You're absolutely right. The lower incisors of hypocalcified teeth are yellowy brown at the incisal tip but look essentially normal at the cervical region. We dissected a whole tooth, determined the mineral content from top to bottom and found that it varied from a low mineral content at the incisal region (55% mineral per volume) to a high mineral content of 85% mineral per volume in the cervical region. There are also variations in the protein content from as little as 1% to as high as 5%. This is why I've presented the range of protein content in each case.

Mann: Do you know anything about the development of the crystals, and can you comment on why they are so irregularly shaped?

Wright: All the teeth we've looked at are fully developed and we've never had the opportunity to look at teeth from different developmental stages, so I can't make any comments about that. However, the crystal shape appears to depend on the type of amelogenesis imperfecta. Hypoplastic cases with a normal mineral content have essentially normal crystallites. In contrast, hypomaturational cases have an uneven distribution of crystallites, some of which appear small and have irregular morphologies. It is extremely difficult to determine the size of crystallites, unless you use sophisticated techniques to establish crystallite orientation. However, we've also treated crystallites with dissociative agents to remove proteins and they appear to get smaller, indicating that they can be associated with substantial amounts of protein.

Mann: Are there any differences in the levels of carbonate?

Wright: In the cases that I have analysed, the levels of carbonate were not significantly different from normal enamel. However, I have not analysed the carbonate content of all the amelogenesis imperfecta cases presented here.

Snead: I would like to follow up on these crystal structure questions and ask for your comments on the concept of amelogenin assembly into 'nanosphere' structures and the notion that an 'A' or 'B' domain in amelogenin is responsible for this assembly, as judged by the yeast two-hybrid system. The amelogenesis imperfecta defects may exhibit specific alterations in their N-terminal 'A' domain or the C-terminal 'B' domain. This may give rise to changes in the assembly of the amelogenin protein and, as a consequence, permit growth along axes other than the c -axis, which is the

dominant characteristic of normal enamel. It is therefore possible that although the protein may be present in the correct amounts, it may not be assembled correctly.

Wright: It may be possible to develop a recombinant, so that enough material could be obtained to look at the assembly of proteins in these cases.

Fejerskov: I would like to point out that there is a range of crystal sizes in fully developed, normal enamel, especially in the surface of erupted teeth. The crystal sizes of enamel obtained from the interior of the tooth, however, are more uniform.

Wright: The results for the hypomaturational enamel are not dependent on the depth of the enamel. The crystallites show marked alteration in size and shape throughout the full thickness of enamel. There is so much protein present in this enamel that treatment with sodium hypochlorite resulted in the enamel coming apart and leaving only small needle-like crystallites. Some of the defects associated with the crystallites may thus be organic or ectopic mineral.

Robinson: If we try to grow rat incisor crystals, which are hexagonal, without the correct supersaturation conditions we can obtain both amorphous and crystalline material, suggesting that it is a mineral problem rather than an organic one.

Wright: I agree that some of the crystallite defects we observe in amelogenesis imperfecta enamel may be mineral defects. The round blobs on some crystallites have a radiodensity that is similar to the mineralized body of the crystallite, implying that this may not be an organic problem.

Deutsch: Have you treated the abnormal crystals with antibodies using the protein-A immunogold method to determine whether proteins are present?

Wright: We haven't done those experiments yet, but we are planning to do them shortly.

Deutsch: I have another, unrelated comment to make. Michael Aldred has found that in a case of X-linked amelogenesis imperfecta, two loci were involved: the amelogenin gene and another unidentified gene that maps to the long arm of the X-chromosome (Aldred et al 1992a).

Aldred: Yes, two loci on the X chromosome are definitely involved.

Snead: In the case of X-linked amelogenesis imperfecta is there compensation from the expression of the Y chromosome amelogenin gene? This is a point that needs to be addressed in such families. There are enough differences in X versus Y amelogenin chromosome/chromosome-coding regions that a Y chromosome-specific transcript or protein could be identified.

Rosenbloom: We don't have a good explanation for this because we don't understand what is regulating the expression of the X and Y chromosome amelogenin genes. There are significant differences between the two proteins, but we don't understand what this means because we don't yet understand the structure-function relationship of amelogenins.

Deutsch: Lagerström et al (1991a) showed a 5 kb deletion in the X chromosome amelogenin gene in a case of X-linked amelogenesis imperfecta. This is an almost natural knockout experiment, since only the first two amino acids of the mature protein are coded for, and yet enamel was present, albeit defective enamel, which

covered the entire tooth. The only possible explanation which comes to mind is that the Y chromosome amelogenin gene compensates for the non-functional X chromosome amelogenin gene.

Lyngstadaas: In experiments with anti-amelogenin hammerhead ribozymes we completely eliminated the expression of amelogenin in live newborn mice for nearly three days (Lyngstadaas et al 1995). The ribozyme was constructed to cut in the 5' end of the amelogenin mRNA to prevent its translation. When this amelogenin 'knockout' was timed to the early stages of enamel development, i.e. early enamel matrix secretion, it resulted in severely disturbed enamel. Enamel structures were hypomineralized with an accumulation of organic material at the prism boundaries, but with general enamel architecture, thickness and distribution intact. This suggests that amelogenin plays an important role in the mineralization of enamel, but is less important in enamel morphogenesis. Deletions in the amelogenin genes should therefore result in mineralization disturbances in the enamel rather than the absence of enamel.

Winter: In the case described by Lagerström et al (1991b) there was a 5 kb deletion spanning exon 3 to exon 7. However, we've shown since that much smaller changes — such as a single base change in exon 5 or exon 6 — have a more significant effect on the phenotype than the 5 kb deletion (Lench & Winter 1995). There must be certain sites in the X chromosome amelogenin gene that are transcriptionally very active.

Robinson: But a small change in amino acid sequence of the order of one or two amino acids can produce considerable damage, in terms of changes to secondary and tertiary structure. This could be more damaging than deleting the entire protein, if there are any compensating mechanisms in operation.

Rosenbloom: I agree. There are many examples where a knockout is less damaging than retaining a protein that has a point mutation because the defective protein can act in a dominant fashion and effectively disrupt all the functional molecules that it interacts with. For example, there are many extracellular matrix protein genetic defects, such as the Marfan syndrome and osteogenesis imperfecta, that exhibit this phenotype.

Aldred: But this situation is by no means as simple as saying "this is the point mutation which causes that particular phenotype" because this does not explain why in some families different individuals have different phenotypes. For example, in one family in which the mutation in the amelogenin gene is known there is a different phenotype in different individuals (Aldred et al 1992b).

Slavkin: An interesting analogy is osteogenesis imperfecta, where different point mutations take place within a general motif of a particular exon, so that the phenotypes are roughly the same but technically they are a result of different point mutations.

Aldred: I haven't used the terms hypomaturation and hypocalcification for a long time simply because I don't know what the difference is in any one individual. I understand that each term can represent one of two extremes, but if one regards the clinical effects as part of a spectrum how does one know where to draw the line, so the

terms are unhelpful. In addition, an obviously hypoplastic enamel can also be hypomineralized, which confuses the issue in terms of the conventional classifications.

Slavkin: Would the syndromic amelogenesis imperfecta numbers be more useful to obtain a molecular understanding?

Wright: No, because most people reserve the term 'amelogenesis imperfecta' for those non-syndromic hereditary conditions that affect only enamel.

Aldred: Yes, but I disagree that this is valid. For example, tricho-dento-osseous (TDO) syndrome is a syndrome in which there is amelogenesis imperfecta. The dental changes in the TDO syndrome are similar or identical to those in one of the forms of amelogenesis imperfecta (Crawford & Aldred 1990). However, in cases of amelogenesis imperfecta with taurodontism, tissues other than enamel may be affected, so when does a case become syndromic? Equally, the case reported by Angelos & Jorgensen (1993) could be regarded as syndromic or as X-linked amelogenesis imperfecta, depending upon one's view of the significance of the subtle hair defects.

Wright: But by those criteria you would call every bone disorder osteogenesis imperfecta. Nomenclature is necessary for communication, and to call everything that has an enamel defect amelogenesis imperfecta is not very useful.

Slavkin: Is there an X-linked dominant or X-linked recessive syndrome in which multiple tissues in the body are affected, including the enamel tissue?

Wright: Coffin-Lowry syndrome is an X-linked disorder that maps close to the amelogenin gene. We've recently been looking at this disorder and, although it's not yet clear, there may be some associated enamel defects.

Snead: There are genetic disorders that have single gene defects and others that have multiple gene defects. Multigene disorders are more difficult to diagnose and categorize because the spectrum of their phenotype can be much broader. As a consequence, if one is trying to do an associative study between a genetic marker within a well-defined pedigree, then the better you define the population under study, the tighter the association will be and the more likely it will lead to a linkage. When talking about a multigene disorder versus a single gene disorder that affects enamel, one has to take into account that although the apparent clinical phenotype may be the same, the number of affected genes that can lead to it may be large. Therefore, one may not find a clear association by searching only for defects in the amelogenin gene. It may be easier at first to look at the range of amelogenesis imperfecta defects in a large population to determine their prevalence, and then move to well-defined clinical phenotypes that have high diagnostic manifestations and potentially informative pedigrees. Submitting such families to associative studies with newly available markers that have been previously subjected to a multipoint analysis, and are therefore very robust, may prove useful. It is more important to speak of well-defined phenotypes, rather than to say that several traits are shared. Ultimately, it will be more useful to think about these phenotypic differences in terms of a specific altered genetic locus and the spectrum of the genetic loci that can contribute to the phenotypic change.

Simmer: I would like to make a point about obtaining a gene-based diagnosis for amelogenesis imperfecta. X-linked amelogenesis imperfecta accounts for about 5% of the total number of cases, and it may be caused by mutations in either of two X-linked genes. If two genes cause only 5% of the cases, there may be 20–40 different genes underlying all of the cases of amelogenesis imperfecta. As amelogenesis imperfecta is limited to disorders affecting enamel in the absence of other symptoms, the underlying mutations are likely to be in genes encoding enamel matrix proteins. This underscores the need to identify the specific constituents of the enamel matrix and to clone the genes encoding them. Using these genes as probes permits a target-gene, rather than a shot-gun, approach in the screening of amelogenesis imperfecta pedigrees. Currently, our knowledge of the constituents of the enamel matrix is in it's infancy. Once we find out what's there, we will be in a better position to develop a genetics-based diagnosis of amelogenesis imperfecta.

Boyde: Are humans the only species that can survive with such a severe defect? Are there no animal models of amelogenesis imperfecta because they could not survive in the wild and would therefore be self-deleting?

Wright: Nobel (1969) reported a lineage of horses that apparently had amelogenesis imperfecta, but other than this you are probably correct in saying that most animals would not survive with these defects.

Bäckman: Some years ago I received a letter from a dog breeder in Denmark who asked me to diagnose the enamel defect in one of her dogs. I am relatively certain that this dog was suffering from some form of amelogenesis imperfecta.

Boyde: But that does not answer the question about the survival of animals in the wild.

Wright: An enamel-less rat has been described by Ishibashi et al (1990). They proposed that it was an example of hypoplastic amelogenesis imperfecta. It had no enamel due to the early death of the ameloblasts.

Boyde: The growth of teeth in the naked mole rat is extremely rapid and the enamel is probably so soft that it is not important functionally, so this could be an animal model.

Butler: What is the effect of osteogenesis imperfecta accompanied by dentinogenesis imperfecta on enamel?

Winter: There is relatively little effect. In dentinogenesis imperfecta associated with osteogenesis imperfecta (type 1) enamel growth is relatively normal, but in heredity opalescent dentine (type 2) the enamel is frequently dysplastic (Sheilds et al 1973).

Aldred: This may be due to a defect in the underlying dentine so that it is unable to support the enamel, rather than in the enamel itself.

Wright: Schimmelpfenning & McDonald (1953) described a condition that involves dentine and enamel with the dentine looking just like dentinogenesis imperfecta. There may be a large variation in terms of the enamel defects in dentinogenesis imperfecta, ranging from a mild discolouration, due to the discoloured and abnormal dentine shining through, to a reduced enamel mineral content and/or structure.

Aldred: You would have to argue that both the genes for dentinogenesis imperfecta and the one for the enamel defect were located adjacent to one another and that the combined defect resulted from a contiguous gene deletion.

Wright: Not necessarily. It could be a secondary effect from an essential signalling event or an early dysfunctional epithelial–mesenchymal interaction that leads a primary dentine defect to affect enamel formation. We don't know the gene defect for dentinogenesis imperfecta type 2.

Zeichner-David: Dentinogenesis imperfecta type 1 has been mapped to chromosome 4, very near to where a case of autosomal inherited amelogenesis imperfecta has been recently mapped.

Snead: cDNA cloning experiments suggest that there are 700 candidate genes, which are expressed during tooth development in the rat. Each of these are potential candidates for different defects in tooth formation (Matsuki et al 1995).

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Extracellular matrix proteins of dentine

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Abstract. Bone and dentine extracellular matrix proteins are similar, consisting primarily of type I collagen, acidic proteins and proteoglycans. Although collagen forms the lattice for deposition of calcium and phosphate for formation of carbonate apatite, the non-collagenous proteins are believed to control initiation and growth of the crystals. Despite this similarity, dentine contains three unique proteins apparently absent from bone and other tissues: dentine phosphophoryn (DPP), dentine matrix protein 1 (DMP1) and dentine sialoprotein (DSP). DPP and DMP1 are acidic phosphoproteins probably involved in the control of mineralization processes. DPP may localize in gap regions of collagen and initiate apatite crystal formation by binding large quantities of calcium in a conformation that promotes this process. Extensive studies have been conducted in our laboratory on the nature, biosynthesis, localization and gene structure of DSP. Immunolocalization studies showed that rat DSP, a 53 kDa sialic acid-rich glycoprotein, was synthesized by young and mature odontoblasts, and by dental pulp cells and pre-ameloblasts, but not by ameloblasts, osteoblasts, chondrocytes or other cell types. The cDNA sequence indicated that DSP was a 366-residue protein with several potential N-glycosylation sites, as well as phosphorylation sites, but that the amino acid sequence was dissimilar to that of other known proteins. Northern blot analysis detected several mRNA species near 4.6 and 1.5 kb, indicative of alternative splicing events. Evidence for two DSP genes was obtained, further complicating this picture. Recent *in situ* hybridization studies utilizing rat and mouse molars and incisors indicated that DSP mRNA was expressed by young odontoblasts and odontoblasts in animals of all ages. Transcripts were also observed in pre-ameloblasts. The expression of DSP mRNA ceased when these cells matured to become secretory ameloblasts. DSP transcripts were not detected in osteoblasts or other cell types. The transient expression in pre-ameloblasts suggests a role of epithelial-mesenchymal interactions in the formation of the tooth.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 107-117

In the formation of bone and dentine, terminally differentiated osteoblasts and odontoblasts secrete an extracellular matrix (ECM) that mineralizes outside the cell. These mineralized tissues are similar in many respects: they both consist principally of collagen fibrils, constructed primarily of type I collagen, with carbonate apatite crystals within and around the fibrils. A great deal of information concerning the

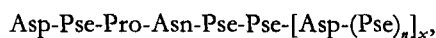
formation of bone and dentine and the role of ECM has been obtained from numerous studies within the past two decades. For reviews see Veis (1993), Linde & Goldberg (1993), Butler & Ritchie (1995) and Robey & Boskey (1996). In addition to collagen, the ECM of bone and dentine contains a number of well-characterized proteins and proteoglycans, referred to as non-collagenous proteins (NCPs), with functions that are under investigation. It has been postulated that some of these NCPs are involved in the initiation of mineralization, and others are thought to control the rate of apatite growth by binding to the surfaces of nascent crystals. Clearly, some of the ECM components of bone and dentine have roles not related to mineralization. For example, several contain Arg-Gly-Asp (RGD) sequences that bind to cell membrane receptors (integrins), possibly resulting in cell attachment and intracellular signalling (Denhardt & Guo 1993). Despite the similarities between bone and dentine ECM, the latter contains at least three NCPs that are unique to dentine and are not found in bone ECM.

Extracellular matrix proteins that are unique to dentine

The three NCPs that appear to be uniquely found in dentine ECM are dentine phosphophoryn (DPP), dentine matrix protein 1 (DMP1, formerly called AG1) and dentine sialoprotein (DSP). It is our intention to overview some of the current information relating to DPP and DMP1, and to provide more details regarding DSP, especially since its cellular expression might relate to the formation of enamel.

Dentine phosphophoryn

DPP was discovered by A. Veis and his colleagues, who have consistently made significant contributions to understanding its structure, tissue localization and potential function. After collagen, DPP is the most abundant protein in dentine. It is characterized by high levels of aspartic acid (*c.* 35%) and phosphoserine (45–50%). The reported molecular mass of DPP varies according to species, ranging from 72 kDa for mouse DPP (MacDougall et al 1985), 90–95 kDa for rat DPP (Butler et al 1983) and 155 kDa for bovine DPP (Stetler-Stevenson & Veis 1983). Several molecular variants of DPP have been detected in rat dentine (Dimuzio & Veis 1978, Butler et al 1983). Although small segments of amino acid sequences are known, the total primary structure for DPP has yet to be reported. A significant contribution was made recently when Crossley et al (1996) utilized a modification procedure for the phosphoseryl residues that allowed them to perform 50 cycles of Edman degradation. The N-terminus for bovine DPP is:



where $n = 1-3$, x = an unknown number of repeating sequences and Pse = phosphoserine.

DPP forms an insoluble aggregate with calcium (Kuboki et al 1979); it binds high levels of Ca^{2+} with a relatively high affinity (Marsh 1989), and this affinity for many molecules of calcium may predict a special biological role in dentine formation. DPP may form extended β -sheets in the presence of calcium.

A sizeable body of evidence suggests that DPP is involved in the initiation of apatite crystal formation in the collagen fibrillar lattice of dentine (reviewed in Butler 1995, Butler & Ritchie 1995). Following its synthesis by odontoblasts, DPP reaches the mineralization front relatively quickly; there, limited numbers of DPP molecules bind to collagen in unmineralized pre-dentine, near the middle of the gap region, where the initial formation of apatite crystals is known to occur. Because of its affinity for calcium, and perhaps due to the conformation assumed by the bound form of DPP, it is able to initiate the formation of plate-like crystals of apatite, associated with channels in the collagen fibrillar network (Landis et al 1993). DPP may also be involved in the control of crystal growth: when larger quantities bind to the (100) face of growing crystals (defined in Addadi et al 1992), the phosphoserine and aspartate groups interact with calcium ions in the lattice, resulting in a reduced rate of apposition of additional calcium and phosphate ions. In this manner, DPP is believed to affect the final 'habit' of dentine apatite crystals (Addadi et al 1992).

Dentine matrix protein 1

DMP1 is a unique phosphorylated dentine protein discovered by screening an odontoblast cDNA library (George et al 1993). The predicted sequence showed a protein with a 16 amino acid leader sequence, followed by a 473 residue secreted portion with a molecular mass (prior to post-translational modifications) of 53 kDa. The preponderance of Asp and Glu residues are indicative of an acidic protein, especially if the more than 100 serine residues that are potential protein kinase substrates are phosphorylated. The ^{32}P -labelled protein that was immunoprecipitated with antibodies to DMP1 yielded a single band with an apparent molecular mass of 61 kDa. The presence of several consensus sequences for N- and O-glycosylation sites suggests that DMP1 is a glycoprotein. Evidence that DMP1 is an odontoblast-specific protein came from *in situ* hybridization studies (George et al 1995), which showed that DMP1 mRNA is present in mature odontoblasts, but not in pulp cells, pre-odontoblasts, stratum intermedium and stellate reticulum. Unexpectedly, transcripts were also detected in secretory ameloblasts. Recently, MacDougall et al (1996) mapped the human DMP1 gene to chromosome 4 in the region 4q21–4q22. Significantly, this locus is in close proximity to that of the dentinogenesis imperfecta type 2 gene. These results confirm and extend the findings of George et al (1994) and suggest some important role for DMP1 in the mineralization process of dentine.

Dentine sialoprotein

DSP was initially isolated from rat dentine and shown to be a unique sialic acid-rich glycoprotein with an apparent molecular mass of 95 kDa; thus, it was called the 95K

glycoprotein (Butler et al 1981). Subsequently, sedimentation equilibrium analysis gave a molecular mass of about 53 kDa (Butler et al 1992). This dentine protein was shown to be rich in aspartic acid, glutamic acid, glycine and serine and to have little or no cysteine. Detailed carbohydrate analysis showed that it contained about 30% carbohydrate, including 9% sialic acid. Based on the molecular weight and carbohydrate content, we predicted that the protein consisted of about 350 amino acids, with approximately 75 monosaccharides present as N- and O-glycosides. The N-terminal sequence was determined by Edman degradation to be:

Ile-Pro-Val-Pro-Gln-Leu-Val-Pro,

a sequence similar to that of osteopontin. Because of the similarities in size and properties of the dentine protein to the bone sialoproteins (osteopontin, bone sialoprotein and bone acidic glycoprotein 75), we renamed it dentine sialoprotein, or DSP.

Next, we performed extensive immunolocalization studies (D'Souza et al 1992, Bronckers et al 1993) using a variety of techniques and tissues. The earliest staining was seen in newly differentiated young odontoblasts concomitant with the secretion of pre-dentine; the immunostaining was prominent in all odontoblasts that were actively forming dentine. Evidence was also found for the presence of DSP within odontoblastic processes and in mineralized dentine matrix. Intense immunostaining was observed within cells and the matrix of dental pulp during dentinogenesis. In the early stages of tooth development, prior to the breakdown of the basement membrane separating epithelial and mesenchymal components, pre-ameloblasts immunostained for DSP (Bronckers et al 1993). We examined many other tissues—including bone and bone cells, cartilage, brain, kidney, liver, blood vessels and muscle—and found them to be devoid of DSP. Certain cells that resembled undifferentiated mesenchymal cells around muscle fascicles surrounding bone were positively immunostained (D'Souza et al 1992). These studies provided evidence that DSP should be considered as a biochemical marker for odontoblasts and that it probably plays an important role in dentinogenesis. Recent ultrastructural immunolocalization studies (A. Nanci & W. T. Butler, unpublished results 1996) detected DSP concentrated in areas around the odontoblastic processes in greater quantities than throughout the dentine matrix; mantle dentine also contained immunogold particles in larger quantities than in the matrix.

Using an antibody to screen a rat odontoblast cDNA library, we obtained a 750 bp clone with a sequence coding for a portion of DSP that included the N-terminus (Ritchie et al 1994). We obtained a second full-length clone by screening with this partial cDNA; the longer sequence coded for a 17 residue leader sequence and a 366 amino acid secreted protein (Fig. 1). The sequence was unique, dissimilar to other reported sequences. The amino acid composition calculated for this latter sequence was identical to that determined for the protein (Butler et al 1992). Additionally, the molecular mass that was calculated from the predicted sequence, when added to that for

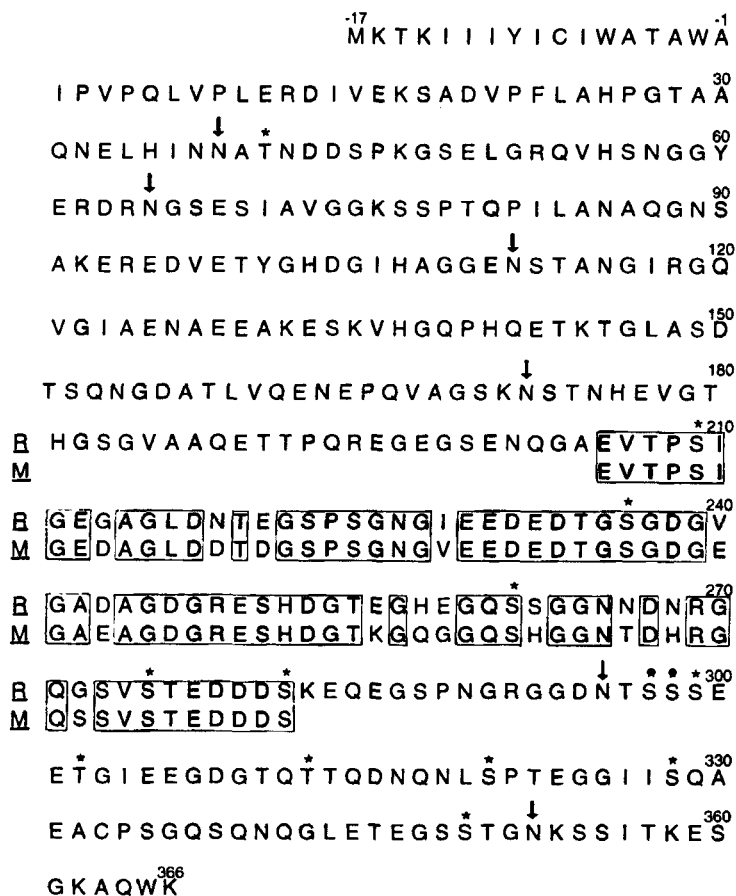


FIG. 1. Complete amino acid sequence for rat (R) dentine sialoprotein (DSP) and partial sequence of mouse (M) DSP, as deduced from the cDNA sequences (Ritchie et al 1994, 1996). In rat DSP, a 17 amino acid leader sequence is followed by 366 amino acids. Arrows denote six potential N-glycosylation sites and asterisks indicate 11 serines and three threonines that are potentially phosphorylated.

the carbohydrate moiety, was about 53 kDa, precisely the size directly determined by sedimentation equilibrium. For these reasons, we concluded that the full-length cDNA corresponded to the complete coding sequence for secreted DSP. Note that the actual molecular weight is substantially lower than apparent sizes obtained by gel electrophoresis.

Northern blot analyses detected multiple DSP transcripts of nearly 4.6 and 1.5 kb in rat incisors and tooth germs (Ritchie et al 1994). No transcripts could be detected in numerous other tissues, consistent with the limited expression of DSP protein seen by immunohistochemistry. The multiple transcripts are probably indicative of differing

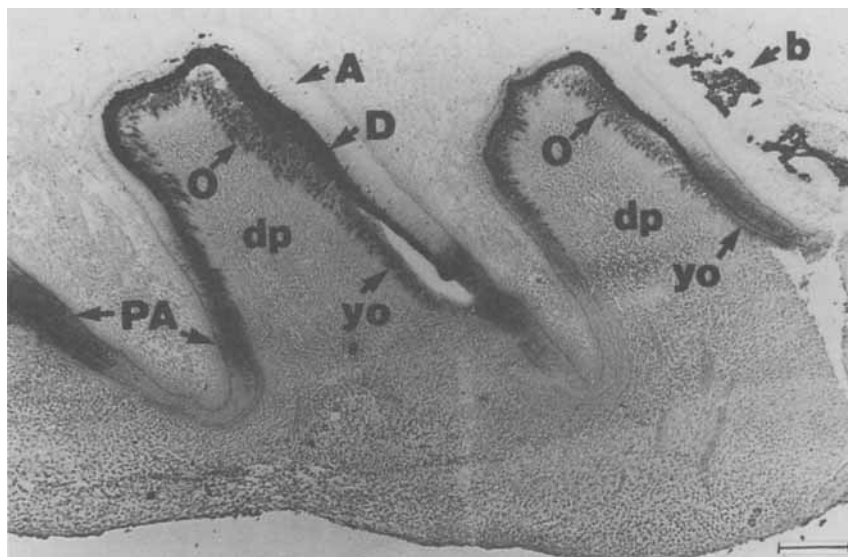


FIG. 2. Immunolocalization of dentine sialoprotein (DSP) in the developing first molar of a two-day-old rat. The avidin-biotin-peroxidase (Vector Labs, Burlingame, CA, USA) method was performed on a hyaluronidase-treated section as described in Bronckers et al (1993). In this section the sequential stages of odontoblast and ameloblast differentiation are observed from the early stages in the apical portions to the later stages in the occlusal region. In this section odontoblasts (O) at all stages express DSP. Young odontoblasts (yo), dentine (D) and pre-ameloblasts (PA) are strongly immunopositive, dental pulp (dp) is weakly positive, and ameloblasts (A) are negative. Bone (b) was not immunostained but displayed a background stain different from that elicited by the peroxidase stain. Bar = 100 μ m.

splicing events in RNA processing. A further complication is that two DSP genes exist (Ritchie et al 1995, Butler & Ritchie 1995). These observations indicate that the expression of DSP by odontoblasts may lead to numerous protein products that could be developmentally regulated and involved in more than one function.

A genomic clone for the rat DSP gene corresponding to the known sequence has been characterized (Ritchie et al 1995). This gene consists of five exons: exon 1 consists of a 50 bp non-coding region; exon 2 is 76 bp long and contains a 19 bp non-coding sequence, the ATG start site, and 57 bp coding for the leader sequence and the first two amino acids of the secreted protein; exons 3, 4 and 5 consist of 87, 930 and 75 bp, respectively, and encode for successive segments corresponding to 29, 310 and 25 amino acids. To date, characterization of the 5' promoter region of the DSP gene has not been achieved.

A mouse partial DSP cDNA has been generated from total mouse RNA by reverse transcriptase PCR techniques using rat DSP oligoprimers (Ritchie et al 1996). The

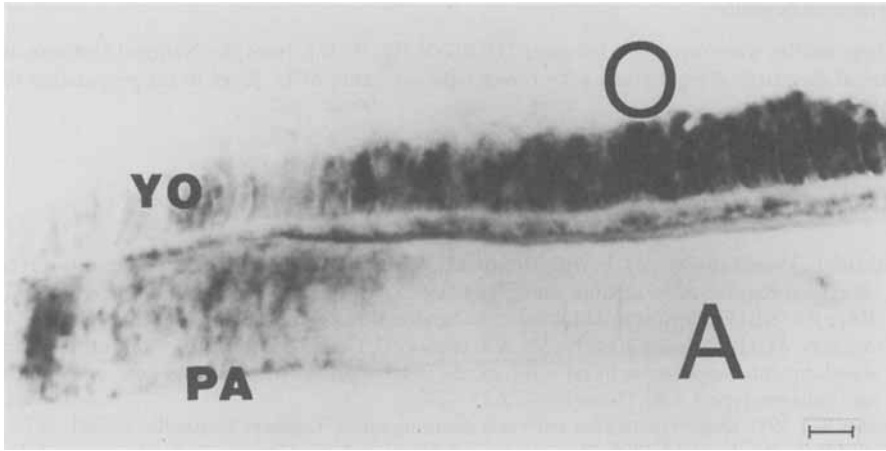


FIG. 3. *In situ* hybridization of the apical region of a two-day-old rat first molar. The section was hybridized with a digoxigenin-labelled dentine sialoprotein (DSP) antisense probe. DSP mRNA expression was detected in young odontoblasts (YO), odontoblasts (O) and pre-ameloblasts (PA). No expression was detected in ameloblasts (A). Bar = 20 μ m.

DNA sequence of the mouse partial DSP cDNA is highly similar to that of the corresponding rat sequence. The similarity of the predicted mouse amino acid sequence to the rat DSP is shown in Fig. 1.

Using rat and mouse riboprobes, we have recently employed *in situ* hybridization techniques to study the expression of DSP transcripts in animals of various ages, from neonatal to adult (H. H. Ritchie, J. E. Berry, M. J. Somerman et al, unpublished data 1996). DSP mRNA was observed in young odontoblasts and in mature odontoblasts in animals of all ages and the pattern of expression was similar to that for immunolocalization experiments (see Figs 2 & 3 for examples). No transcripts were detected in dental pulp, enamel, bone or any other oral tissues examined. The reason for the lack of expression of DSP mRNA by pulp cells is unknown; immunohistochemical studies showed the presence of DSP protein in these cells and possibly in the surrounding matrix (D'Souza et al 1992).

Significantly, pre-ameloblasts in developing teeth expressed DSP (Fig. 2) and DSP mRNA (Fig. 3), confirming our earlier immunohistochemical observations (Bronckers et al 1993). The *in situ* hybridization results rule out the possibility that pre-ameloblasts were immunostained because the antibodies reacted with a product antigenically related to DSP. The synthesis of DSP and DSP mRNA by pre-ameloblasts and young odontoblasts (Figs 2 & 3) indicates that DSP is involved in some phase of the reciprocal epithelial-mesenchymal signalling that accompanies the developmental process of teeth.

Acknowledgements

These studies were supported by grant DE-05096 (W. T. B.), from the National Institute of Dental Research. We gratefully acknowledge the assistance of L. Jones in the preparation of this manuscript.

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DISCUSSION

Zeichner-David: During the 1996 meeting of the International Association of Dental Research in San Francisco Mary MacDougall mentioned that dentine sialoprotein (DSP) is also present in bone. Have you observed this in your experiments?

Butler: We've looked very carefully at the expression of DSP mRNA and protein in a number of tissues including bone and we've never detected expression in bone.

Snead: We are interested in regulated gene expression and how it occurs in regionalized sheets of cells. I do not believe that all ameloblasts are equal, and your results suggested that amelogenin was expressed not just in the pre-ameloblasts but also on the balancing cusps. There was not a high level of amelogenin expression where the enamel was less thick, and it is possible that if you had looked at the enamel-free zones you would have observed sites at which amelogenin is expressed at lower levels than at the transition between pre-ameloblasts and ameloblasts. Therefore, your results could be re-interpreted to suggest that rather than there being a positive up-regulator of amelogenin expression or ameloblast differentiation, there are areas in which the phenotype is less well expressed and amelogenin expression is down-regulated or inhibited.

Butler: I welcome your comments. I would, however, emphasize that one of the most striking aspects of our study is the demonstration that pre-ameloblasts suddenly switch on the expression of DSP mRNA and protein and that this expression is abruptly suppressed or switched off in ameloblasts.

Snead: I am concerned about how a 4.6 kb mRNA transcript is obtained from a 4.0 kb hybridization band on a Southern blot, and about the relationship between the mRNA and its genomic counterpart. You may need to use another probe, perhaps one specific to the 3' untranslated region, which is more selective in terms of detecting isoforms.

Boyde: What is the pattern of labelling on the lingual side of the incisor?

Butler: The odontoblasts are labelled strongly for DSP mRNA. Ameloblasts are only present on the labial side of the rat incisor.

Jones: Am I correct in saying that the peritubular dentine is not labelled?

Nanci: Rats do not have a well-defined peritubular dentine layer. Bill Butler showed that there is a diffuse labelling of DSP throughout the intertubular dentine matrix and, in some cases, a concentration around the tubules. Mantle dentine also appears to be somewhat more immunoreactive.

Diekwisch: There may be some labelling of the pre-dentine but the labelling of the odontoblasts is so much stronger that it is difficult to tell. I believe that pre-ameloblasts are labelled positively, especially towards the beginning of root formation. It is interesting that odontoblasts and ameloblasts, or pre-ameloblasts and pre-odontoblasts, have a similar protein expression pattern even though they are derived from different tissues, i.e. ectodermal and mesenchymal tissues. This similarity in expression patterns can also be observed by analysing enamel protein expression patterns; for example, tuftelin and amelogenin. An explanation for this phenomenon may be protein translocation.

Simmer: Your comments about the common N-termini of the secreted proteins may be over-emphasized. Exon 2 contains the start codon, the coding region for the signal peptide, and it encodes the first two residues of the secreted protein. Secreted amelogenins usually have Met-Pro-Leu at their N-terminus, with rabbits having Leu-Pro-Leu and opossum Ile-Pro-Leu. Therefore, the features you mentioned may be a common phenomenon and potentially related to the specificity of the signal peptidase.

Butler: There are more similarities than just that. The proteins are all about the same size, they are rich in sialic acid, they are single chain acidic proteins and the chromosomal loci are the same.

Sharpe: It is important to identify whether there is a second gene because you may be detecting both genes, if they exist. Therefore, the odontoblasts and pre-ameloblasts may be expressing different genes.

Zeichner-David: You mentioned that dentine phosphophoryn (DPP) might initiate the nucleation of hydroxyapatite crystals, but what are the functions of dentine matrix protein 1 (DMP1) and DSP?

Butler: The functions of these two proteins are not known at the moment, so I can only speculate. DSP is secreted by pre-ameloblasts, and then by young odontoblasts and odontoblasts. The reciprocal expression first by pre-ameloblasts and then by young odontoblasts known to be important in epithelial-mesenchymal interactions suggests some role in cell signalling. *In vitro*, DSP inhibits mineralization but to a lesser extent than osteopontin. This observation implies that once the crystals are growing, DSP might be one of the proteins binding to the crystal surface and slowing down crystal growth. However, there is no direct evidence to support this idea.

Veis: I would like to make a few relevant comments. Jerry Dillon in my laboratory, in collaboration with Stephen Weiner at the Weizmann Institute, is currently looking at proteins in human peritubular dentine. There is a small amount of extracellular matrix protein, much of which has not been identified yet, and some DPP.

We have also been studying the sequence of DPP in collaboration with Anne George. Within the 300 amino acids at the C-terminus there are a number of

Asp-Ser-Ser repeats, in which the serine residues are putative phosphorylation sites (A. George & A. Veis, unpublished results 1995). We have shown that many of the phosphoproteins in the extracellular matrix are phosphorylated by casein kinase type II (CK2), but these Asp-Ser-Ser repeats are not direct targets of CK2 (Wu et al 1992). However, phosphorylation of a few serine residues in classical substrate sites at the C-terminus allows for the hierarchical phosphorylation of all of the remaining serine residues. This phosphorylation cascade causes this part of the molecule to become extremely anionic. Modelling studies suggest that ordered arrays of carboxyl and phosphate groups create calcium ion binding domains organized in exactly the same fashion as Addadi & Weiner (1985) proposed would enable the maximum binding to hydroxyapatite surfaces.

Zeichner-David: I agree that CK2 might further the phosphorylate DPP. However, we have demonstrated that CK2 cannot phosphorylate DPP directly, and this is also true for CK1. There is a different kinase present in the odontoblasts which is capable of initiating the phosphorylation of dephosphorylated DPP (dephosphorylated with potato acid phosphatase).

Veis: Phosphophoryn is not a substrate for messenger-dependent kinases but the catalytic subunit of protein kinase A will phosphorylate dephosphorylated phosphophoryn. In turn, dephosphorylated phosphophoryn is not a substrate for CK2, but after limited phosphorylation with protein kinase A, CK2 will go on to phosphorylate its 'new' substrates. CK1 will then be active. The occurrence of this progressive set of reactions is why I have used the term 'hierarchical phosphorylation'.

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Amelogenin proteins of developing dental enamel

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Abstract. The amelogenins of developing dental enamel are tissue-specific proteins, rich in proline, leucine, histidine and glutamyl residues, and synthesized by the ameloblast cells of the inner enamel epithelium. These proteins comprise the bulk of the extracellular matrix that becomes mineralized with a hydroxyapatite phase to become the mature enamel. Examination of the amino acid sequences of amelogenins from a range of mammals shows a high degree of evolutionary sequence conservation, suggestive of specialized function. Recently it has been shown that multiple amelogenin components, observed in the matrix, arise both by a sequence of post-secretory proteolytic processing and by the expression of alternatively spliced mRNAs generated from the amelogenin gene(s) that are located on the sex chromosomes. Although the function of these amelogenins in enamel biomineralization is unknown, physico-chemical studies of recombinant amelogenins have shown that they undergo a self-assembly process *in vitro* generating supra-molecular 'nanosphere' structures, and recent observations *in vivo* point to a functional role for the nanospheres in the ultrastructural organization of the secretory enamel matrix, conducive to the organized development of the earliest mineral crystallites.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 118-134

The soluble protein contained no hydroxyproline or hydroxylysine and only moderate amounts of glycine, indicating that it was not a collagen. Its composition showed several distinctive characteristics but was unlike any other known protein.

K. A. Piez, 1960

The amelogenin proteins are tissue-specific components of the extracellular protein matrix of developing dental enamel, and they are synthesized and secreted by the ameloblast cells of the inner enamel epithelium. This chapter will review some recent features of the biochemistry, and molecular and structural biology of these proteins in relation to their putative function in dental enamel biomineralization and amelogenesis.

Enamel biomineralization: background

The biomineralization of enamel with crystals of a carbonated hydroxyapatite occurs extracellularly within a secreted protein matrix. The protein components of this matrix include the amelogenins and other 'non-amelogenin' proteins. The term 'amelogenin' was originally coined by Eastoe (1965) for the principal proline-rich enamel matrix protein. Subsequently, on the basis of a sequential guanidine/guanidine-EDTA extraction procedure, Termine et al (1980) identified a minor protein fraction (5–20%) which they then termed 'enamelin'. This non-amelogenin (enamelin) component has since been shown to include enamelines, tuftelin, ameloblastin (Krebsbach et al 1996), serum albumin and proteinases, although the origin of the serum proteins is thought to lie in post-mortum contamination. Apart from some of the proteinases (Moradian-Oldak et al 1994a), a definitive biological function for these matrix proteins has not been established.

Enamel biomineralization begins at the dentine–enamel junction (DEJ) where tuftelin, ameloblastin and/or enamelin proteins are deposited on a pre-mineralized collagenous dentine. Initial enamel crystallites form as needles or ribbons of mineral generally orientated normal to the DEJ. Whether these crystallites are initially of octacalcium phosphate or of apatite and whether they are nucleated *de novo* or originate from the dentine mineral remains controversial and is not discussed further in this chapter. Subsequent to mineral nucleation, the ameloblast cells secrete a layer of matrix dominated by the amelogenin proteins. This secretion appears to continue as the ameloblasts 'pull-away' from the DEJ. The initial enamel crystallites elongate in their *c*-axial directions to produce enamel ribbons of 1–2 nm thickness by 5–10 nm deep and apparently extending through the full thickness of the secreted matrix, perhaps up to 1000 μm , depending on the species. Subsequently, during the 'maturation stage' of development, a massive loss of matrix protein and water occurs concomitant with the rapid growth of the crystallites, now enlarging in the *a*- and *b*-face dimensions to eventually yield the orientated and exceptionally large mineral crystals that characterize the mature enamel prisms. This process occurs at different rates according to the species, but the temporal and cellular factors that control the synthesis, secretion, processing and eventual removal of the matrix proteins are poorly understood. Nevertheless, evidence for the functional role of the amelogenin proteins in enamel development has been provided by studies of the genetically related condition of X-linked amelogenesis imperfecta, in which deletions in the amelogenin gene have been described (Lagerström et al 1990, Lagerström-Fermér et al 1995) and in the recent study (Lyngstadaas et al 1995) where the *in vivo* use of a ribozyme specific for the amelogenin mRNA led to a gross disturbance in normal enamel development. (For a general review of enamel biomineralization see Simmer & Fincham 1995.)

Amelogenin primary structure and homology

Amelogenin proteins isolated from a range of species have been found to have molecular weights within the range of 7–25 kDa, although some substantially higher

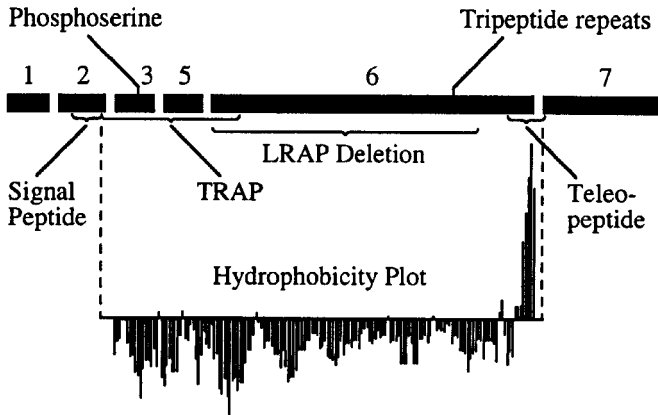


FIG. 2. Relationships between amelogenin protein and gene structures. Amelogenin exons are shown as numbered bars. The principal amelogenin secretory product is translated from a mRNA lacking exon 4. The signal peptide contains 16 residues encoded by exon 2. A hydrophobicity plot using the Hopp and Woods scale is shown below. Note the teleopeptide is the only hydrophilic segment. TRAP is the tyrosine-rich amelogenin polypeptide containing the first 44 or 45 amino acids of the secreted protein. LRAP is the leucine-rich amelogenin polypeptide formed by the deletion of most of exon 6 during splicing and cleavage of the teleopeptide following secretion. The positions of tripeptide repeats (found in the bovine and opossum amelogenins) are shown.

function across evolution. A comparison of amino acid sequences for these highly conserved N- and C-terminal motifs is shown in Fig. 3.

Numerous earlier studies of developing enamel proteins employing electrophoretic characterization revealed a complex pattern of amelogenin components and it had been assumed that this pattern essentially represented the products of the post-secretory proteolytic processing of a single, primary, secreted amelogenin. Gibson et al (1991) demonstrated that in the cow a complex pattern of amelogenins could also arise by the expression of multiple amelogenins resulting from the alternative splicing of the primary RNA transcript, creating a family of mRNAs in which certain exons, or parts of exons, were skipped. In humans amelogenin is expressed by copies of the gene located on both the X and Y chromosomes (Salido et al 1992).

These observations also provided an explanation for earlier studies of bovine amelogenins (Fincham et al 1981), which had identified two polypeptides (5–6 kDa) apparently each representing the N-terminal sequence of a larger amelogenin precursor. While one of these polypeptides (TRAP) was identified as being the N-terminal of the principal bovine amelogenin, the other polypeptide (i.e. leucine-rich amelogenin protein [LRAP]) could not be related to any known bovine amelogenin sequence.

This puzzle has now been explained through the identification of an alternatively spliced mRNA coding for a 59 residue polypeptide (Gibson et al 1991) which,

A

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Bov: MPLPPHPGHPGYINF SYEVL TPLK WYQ SMIRHPYPSYGYEPMGGW ...
Hum: MPLPPHPGHPGYINF SYEVL TPLK WYQS-IRPPYPSYGYEPMGGW ...
Mus: MPLPPHPGSPGYINLSYEVL TPLK WYQ SMIRQPYPYPSYGYEPMGGW ...
Rat: MPLPPHPGSPGYINLSYEVL TPLK WYQ SMIRQPYPYPSYGYEPMGGW ...
Pig: MPLPPHPGHPGYINF SYEVL TPLK WYQ NMIRHPYTSYGYEPMGGW ...
Pos: IPLPPHPGHPGYINF SYEVL TPLK WYQ SMMRQQYPSYGYEPMGGW ...
Rab: LPLPPHPGHPGYINF SYEVL TPLK WYQ SI ...

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B

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Bov: ... PDLPLEAWPATDKTKREEVD
Hum: ... PDLTLEAWPSTDKTKREEVD
Mus: ... PELPLEAWPATDKTKREEVD
Rat: ... PELPLEAWPATDKTKREEVD
Pig: ... PDLPLEAWPATDKTKREEVD
Pos: ... PD---EAWPATDKTKREEVD

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FIG. 3. Conservation of amelogenin primary structure. Differences from the bovine sequences are shown in bold. Serine 16 is thought to be phosphorylated in all cases. (A) N-terminal (i.e. tyrosine-rich amelogenin polypeptide) sequences. (B) C-terminal sequences.

following C-terminal proteolytic processing, would yield the LRAP structure originally sequenced (Fincham et al 1981). Subsequently, it has been shown that a pattern of alternative splicing of amelogenin transcripts is common to all species studied, and it can be expected to contribute to the generation of the amelogenin complex observed in the developing enamel extracellular matrix (Lau et al 1992, Simmer & Snead 1995). Recent results, employing mass spectrometric analyses of amelogenin preparations from a range of species, have identified some fragments arising from these alternatively spliced constructs at the protein level (Fincham & Moradian-Oldak 1996), although definitive data on the levels of expression and their temporal and developmental control are generally lacking. In summary, it is now clear that a strikingly high degree of amelogenin sequence conservation exists between

mammalian species and that, through the alternative splicing of the primary RNA transcript, multiple (nine in the case of the mouse) amelogenins may be expressed and secreted into the extracellular matrix. (For a review of the molecular biology of the amelogenins see Simmer & Snead 1995.) However, the biological significance of these processes to enamel biomineralization is presently obscure.

Amelogenin secondary and tertiary structures

Early attempts to obtain structural information for the proteins of developing enamel employing X-ray diffraction yielded controversial results, generally interpreted to show a poorly defined α , β or cross- β secondary structure (Fincham et al 1965, Bonar et al 1965). Subsequently, a nuclear magnetic resonance (NMR) study of isolated bovine enamel proteins (Termine & Torchia 1980) suggested that most of the matrix proteins had little defined secondary structure with their molecules being in rapid isotropic molecular motion, and a study of mineral-bound enamel proteins (enamelins) suggested that the poor cross- β diffraction pattern originally reported might arise from these enamel components rather than from the amelogenins (Traub et al 1985). Recent 2D and 3D NMR studies of a recombinant amelogenin appear to confirm these earlier observations, whereas circular dichroism spectra show evidence for an inverse temperature transition (Urry 1995), indicating an increased degree of secondary structure with elevation in temperature (A.G. Fincham & V. Renugopalakrishnan, unpublished observations 1995). Collectively, these observations suggest an anomalous situation in which a matrix composed largely of an 'unstructured' protein appears to facilitate the formation of a highly ordered mineralized structure.

Amelogenin post-translational modifications

Early studies of the bovine amelogenins reported that these structures were phosphorylated, with perhaps up to three phosphoserines per molecule and that such phosphorylated groups, together with side-chain glutamyl carboxyls, might act to chelate calcium ions specifically (Glimcher 1979). Confirmation of this phosphorylation proved difficult, but studies employing mass spectrometric techniques have now established the presence of a single phosphoserine residue located at position 16 in pig amelogenins (Fincham et al 1995), and mass spectrometric data derived from other species can only be matched to the known amino acid sequences assuming such a single phosphoserine (Fincham & Moradian-Oldak 1996). This locus (Ser16) is within the highly conserved TRAP motif (see Fig. 2), and it appears likely that it has a functional significance. Glycosylation of amelogenins has been postulated from time to time, but the recent mass spectrometric data referred to above appear to rule out glycosylation, at least in the limited range of species so far studied (see also Fincham et al 1991). As secreted proteins, the amelogenin mRNAs code for a protein containing a leader (signal)

sequence. In the cases where cDNA sequences have been obtained, amino acid sequence data for the leaders are also available (data not shown) and reveal a high level of homology.

Physico-chemical properties

The cloning of the mouse amelogenin (Snead et al 1985, Lau et al 1992) and the current availability of a recombinant analogue of the mouse 180 residue amelogenin (Simmer et al 1994) have permitted a closer examination of the physico-chemical properties of the molecule than hitherto possible.

The recombinant amelogenin (M179) comprises 179 residues, lacking only the N-terminal methionine and the phosphorylation of serine 16, as compared to the *in vivo* mouse 180 residue molecule. The M179 molecule has a computed pI value of 6.6 and the protein is found to have a minimum solubility of 300 mg/ml (pH 7.0, 50 mM phosphate at 25 °C; J. Tan, personal communication 1996). The solubility of the protein is strongly influenced by pH, ionic strength and temperature, tending to a minimum under physiological conditions. This property places limitations on experimental manipulation of the protein for functional or structural studies and, in most cases, researchers have been forced to diverge from physiological solution conditions in such work. For example, Aoba et al (1990), in examining the apatite-binding properties of porcine amelogenins, were caused to work at 4 °C and a pH of 6.0 in order to obtain a sufficient protein concentration, and Goto et al (1993) made circular dichroism spectral studies of amelogenins working at a pH of 5.0–5.3. These solubility limitations are cause for concern in the interpretation of the physiological significance of such data, and it is also clear that small changes in either local pH or ionic strength within the extracellular matrix *in vivo* can be expected to have a profound effect on local amelogenin solubility.

Quaternary structure and amelogenin function

Recognizing that 'structure determines function', several studies have sought to relate the known structural features (essentially the primary structure) to the properties of the amelogenin molecule and its putative function in enamel biomineralization. This exercise is continually frustrated by the absence of definitive data on secondary or tertiary structure. Travis & Glimcher (1964) proposed a structural role for the enamel protein(s) in which a compartmentalized matrix of micro-channels was created determining both crystal and prism boundaries. Smales (1975), in a rarely quoted study of developing rat enamel, reported the presence of globular structures that appeared to be helically arranged around the developing enamel crystallites. Based on their physico-chemical studies of amelogenins, Aoba & Moreno (1989) postulated a model in which the initially secreted insoluble amelogenin was in equilibrium with bound amelogenin adsorbed onto specific crystal faces, thus controlling their growth. Subsequently, they argued, proteolytic cleavage of the hydrophilic C-terminal

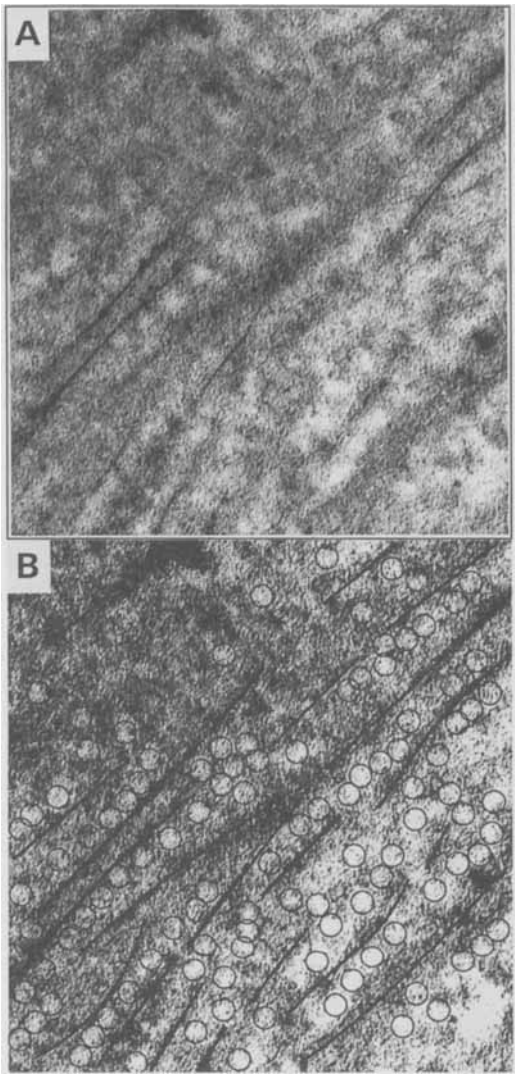


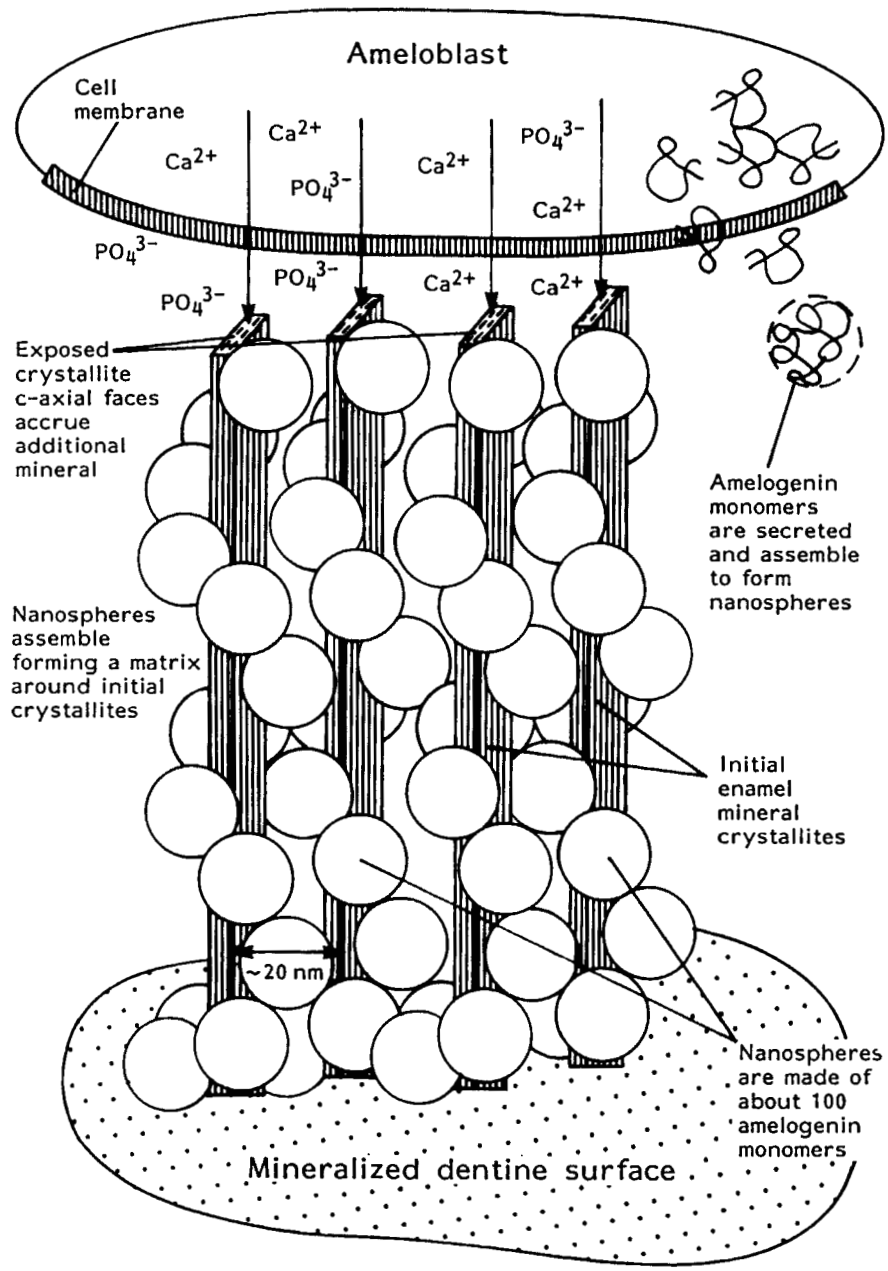
FIG. 4. Identification of nanosphere structures associated with the initial stages of enamel crystallite formation. (A) Transmission electron micrograph of developing mouse enamel showing initial crystallites 2–3 nm in width with beaded rows of nanosphere structures aligned in-between and beside the crystallites. (B) Scanned image of plate A superimposed with a cartoon showing alignment of crystallites (lines) and nanospheres (circles). The circles are approximately 20 nm in diameter.

domains yielded soluble amelogenin which was then translocated from the mineralizing site. Fincham et al (1991) proposed a similar scheme, but suggested that the bi-polar properties of the amelogenin primary structure (hydrophilic-hydrophobic) would cause the alignment of amelogenin monomers to create aggregate structures which associated with the *a*- and *b*-faces of the developing apatite crystals, promoting *c*-axial growth.

Most recently, the availability of the recombinant mouse amelogenin M179 (Simmer et al 1994) has permitted structural studies of amelogenin employing atomic force microscopy, transmission electron microscopy (TEM) and dynamic light scattering techniques (Moradian-Oldak et al 1994b, Fincham et al 1994). This multifaceted approach has shown that, in solution (e.g. pH 7–8 and ionic strength 0.02–0.05) the recombinant amelogenin appears to self-assemble into quasi-spherical, supra-molecular structures (nanospheres) of the order of 18–20 nm in diameter and calculated to be composed of some 100 amelogenin monomers. (For a review see Moradian-Oldak et al 1995.) These novel observations are in keeping with earlier studies employing freeze-fracture scanning electron microscopy of the developing enamel matrix (Robinson et al 1981, Bai & Warshawsky 1985) in which colinear spheres of organic material were reported within the matrix. Although it has been suggested that such structures may be artefactual products of the fixation procedures used (Lyaru et al 1984), the results obtained using the recombinant amelogenin do not support that view and argue for the presence of unique amelogenin quaternary nanosphere structures as a functional moiety in enamel biomineralization.

Developing this thesis further, it has recently been shown that the amelogenin nanospheres, such as elastin (Franzblau & Faris 1981), appear as electron-lucent structures when contrasted by conventional (lead citrate, uranyl acetate) methods for TEM, and in a survey of such TEM images of developing enamel from mouse, cow and hamster, Fincham et al (1995) have suggested that the electron-lucent structures seem to be aligned in 'beaded rows' with the long axes of the earliest developing mineral crystallites (Fig. 4). These observations have led to a new model for amelogenin function in which nanospheres, created by the self-assembly of freshly

FIG. 5. (*opposite*) Hypothetical model for nanosphere-mediated control of initial enamel mineral spacing and growth. Initial enamel crystallites (1–3 nm thick) are nucleated at the mineralized dentine surface (bottom) and become spaced at about 20 nm apart by nanospheres which self-assemble following secretion of amelogenin monomers by the ameloblast (top). With progressive secretion of amelogenin, a matrix of nanospheres is formed around the developing crystallites, (centre) preventing lateral (*a*-*b*-face) crystal growth and crystal-crystal fusions. The unobscured crystal faces adjacent to the ameloblast cell membrane grow in the *c*-axial direction by accretion of calcium and phosphate ions transported by the ameloblast. This dynamic process is conjectured to generate an array of thin discrete mineral crystallites extending through the full thickness of the enamel matrix from the dentine-enamel junction to the ameloblast membrane. Subsequently, proteolytic processing of the amelogenin nanospheres creates conditions permissive of crystallite *a*- and *b*-face growth leading to the creation of the highly ordered enamel prism structure.



secreted amelogenin monomers, form spherical quaternary structures having the anionic C-termini externalized. It is postulated that these nanospheres (about 20 nm in diameter) are deposited around and in-between the developing mineral ribbons (about 2×10 nm in width and thickness), preventing early lateral (*a*, *b*-face) mineral accretion, crystal-crystal fusions and crystal fractures, while leaving the *c*-axial crystal surface exposed to calcium and phosphate ions, transported into the matrix space immediately adjacent to the Tomes' processes of the secretory ameloblasts. This spatial configuration is supported by *in vivo* observations which have shown that the initial mineral crystallites are indeed spaced some 20 nm apart (Fincham et al 1995, Diekwisch et al 1995), and it is thought that these thin initial mineral crystallites may extend through the full 100–200 μ m thickness of the secretory matrix.

The model depicted in Fig. 5 postulates that the quaternary amelogenin structures act as anionic beads creating a structured matrix that promotes the formation of an ordered array of thin (2–3 unit cells thick) mineral crystallites throughout the full thickness of the developing enamel. This structure being in place, proteolytic processing of the exposed C-terminal sequences leads to eventual disruption of the initial protective nanosphere environment then facilitating lateral mineral growth and, possibly, generating amelogenin-derived polypeptide products which provide feed-back signalling to the secretory ameloblasts controlling further amelogenin synthesis and secretion (Robinson et al 1995).

Support for this new model of amelogenin function in enamel biomineralization is derived from the earlier observations on matrix ultrastructure (Smales 1975, Robinson et al 1981, Warshawsky et al 1984), the data now available for the quaternary structures formed by the recombinant amelogenin *in vitro* and the observations *in vivo* of the electron-lucent nanosphere structures, together with the data on initial crystallite spacing.

Acknowledgement

This work was supported, in part, by the National Institutes of Health, National Institute of Dental Research grant DE-02848.

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DISCUSSION

Nanci: I have been examining, in collaboration with Alan Fincham and Jim Simmer, the appearance of purified M179 recombinant amelogenin and comparing it with the texture of proteins *in situ* in the enamel matrix. We mixed M179 protein with the

hydrophilic LR White acrylic resin (London Resin Company, Ltd) and sections were prepared for colloidal gold immunolabelling with the anti-M179 antibody. The sections showed long filamentous structures decorated with numerous gold particles, indicating that the filaments consisted of amelogenin. Based on the size of the colloidal gold used, the diameter of these filaments was estimated as being about 10 nm. In another experiment, the protein was fixed with 1% glutaraldehyde, the fixative we routinely use for immunolabelling of mineralized tissues. In this case, the protein appeared as a finely granular material showing a homogeneous immunolabelling. These results led us to conclude that experimental conditions and, hence, the microenvironment in which M179 is found *in vivo* can affect the conformation this protein assumes. *In vitro* results from experimental conditions that do not reproduce exactly the *in vivo* environment must therefore be taken with great caution. To address this issue, we are planning to resuspend the M179 protein in the enamel fluid described by Aoba & Moreno (1987) at temperatures as close as possible to those of the body. We feel that this will allow us to observe the conformation of the protein in more physiological conditions. Intermixing of the protein with isolated enamel crystal will also be important in determining the behaviour of the protein in the presence of mineral.

Snead: This description of the filaments reminds me so much of intermediate filaments and microtubules that it's almost impossible not to raise the notion of a construct for enamel formation *vis-à-vis* the protein tubulin. Tubulin assembles from a monomeric protein into very high molecular weight structures and it is extremely dynamic in the cytoskeleton.

Oldak: Tony Nanci's results regarding the formation of the filaments by the lyophilized M179 protein may be strong evidence in favour of the idea that the presence of water molecules is another critical parameter for the formation and stability of the nanospheres. Using dynamic light scattering, we have shown (Moradian-Oldak et al 1994) that each nanosphere is composed of approximately 100 molecules. This number was calculated from the hydrodynamic radius of the particles that contain the M179 molecules, without taking into account the number of water molecules. The actual number of M179 in one nanosphere may well be much less if there are water molecules incorporated into the nanosphere structure.

Slavkin: Would this suggest that the structures are hollow?

Oldak: They may be hollow or they may be porous.

Slavkin: Alan Fincham has described that in typical preparations the position of amelogenins are electron lucent. However, Toni Nanci has just described that lyophilized amelogenin is no longer electron lucent. Does anyone have comparable results? Does elastin have the same property?

Fincham: We have used conventional contrast reagents and phosphotungstate, which is a classical negative stain (Fincham et al 1994). With this procedure we observed negatively stained objects on the spray preparation. We observed the same staining pattern, however, when we used uranyl acetate/lead citrate. We read Franzblau & Faris' work on elastin, who positively stained elastin with palladium chloride

(Franzblau & Faris 1981), and we also applied this method to amelogenin. The result was that everything was black. Therefore, with this method we observe the positive staining of the recombinant amelogenin, but with both uranyl acetate and phosphotungstate we observe a negative staining effect.

Boyde: That is because you are lyophilizing with a surplus of staining material. You would expect the same result with any salt solution that you lyophilize, where protein would remain as 'holes' in a material that is denser.

Diekwisch: But the staining of *in vivo* fixed material with palladium chloride and uranyl acetate, which are both considered to be negative stains, produces differences in pattern. Obviously, the terms positive and negative stains are relative. Our results indicate that 'stippled materials' are labelled positively using palladium chloride in those areas that appeared electron lucent with uranyl acetate.

Mann: You mentioned that the amelogenin nanospheres are restricted to a diameter of 20 nm, which represents 100 molecules. How is this achieved?

Fincham: Let me first summarize what we have done. We have identified what we refer to as nanospheres by three independent imaging techniques: atomic force microscopy; transmission electron microscopy; and dynamic light scattering. All three techniques reveal structures with a diameter of the order of 20 nm. Dynamic light scattering experiments have suggested that these nanosphere structures contain about 100 monomeric molecules. We therefore envisaged that there must be some sort of subunit in order to generate a structure with so many molecules. Our recent results suggest that a bimodal distribution is present. Analysis of these distribution data predicts that 'classic' nanospheres of about 20 nm diameter are present and that there is also a subpopulation of particles that have a diameter of 4–5 nm, which would accommodate about 4–6 amelogenin monomers. This suggests that substructures, comprising 4–6 amelogenin monomers, are somehow assembled together. Moreover, it is conceivable that the filaments which Tony Nanci described earlier are made up of these smaller structures.

Veis: In the tissue there is a mixture of amelogenins of different molecular masses. Are they all contained within a single nanosphere or are the smaller amelogenins excluded, so that there are some amelogenins in the fluid around the nanospheres which are not contained within them?

Fincham: This is not known, but I speculate that the 20 nm nanospheres comprise a single larger amelogenin and that smaller amelogenins make smaller nanospheres.

Nanci: These proteins can be cleaved by proteinases, which raises the possibility that the 20 nm nanosphere is a dynamic and possibly also transitory structure.

Fincham: Absolutely. The model I'm putting forward suggests that the anionic C-termini will be externalized, and we know that the proteases generally attack that C-terminal. Therefore, the exposed C-terminal hydrophilic sequences are in exactly the correct positions to be cleaved by the proteases. We are also arguing that such proteolysis will change the structure of the matrix.

Slavkin: When the transmission electron microscope was first developed, newly secreted enamel material was described as electron-dense granular material. At that time it wasn't

clear whether the dense or the non-dense part of the image was relevant but, significantly, the particles were in the 10–25 nm range, which supports the assembly idea.

Robinson: I would like to comment on the possibility that negative feedback from degradation fragments is occurring, and that this stops enamel secretion. We calculated that the concentration of enamel protein degradation products increases during secretion and reaches 50% of the total matrix protein at the point where matrix ceases to be deposited (Robinson & Kirkham 1984). We did this calculation for four or five species. With regard to matrix structure, your model uses the parent 27 kDa recombinant protein at the surface. However, the bulk of the matrix is composed of the 20 kDa processed product and low molecular weight material, i.e. your model describes the possible surface structure and not the bulk of the underlying material. We don't know therefore whether the 20 kDa protein in combination with other fragments forms the same kind of structure.

Fincham: That is true, and we also don't know what the implications are for the multiple alternatively spliced amelogenin variants.

Yamada: It would be interesting to examine whether aggregation is still observed if the C-terminus is deleted. Is it possible that *in vivo* other proteins are incorporated within this aggregated structure?

Fincham: Yes it is possible, but we are speculating that because the amelogenins are the dominant proteins during that phase of development, the structures contain almost exclusively amelogenin.

Yamada: A minor component could still regulate the size.

Oldak: We have investigated the aggregation properties of the mutant M166, which lacks the hydrophilic C-terminal 13 residue sequence of M179 (Moradian-Oldak et al 1994). We have found that M166 was not soluble at pH 7.4–8.0, conditions under which we obtained the large monodisperse M179 aggregates (nanospheres). Only at pH 5.6 and ionic strength 0.05 were aggregates with a high level of polydispersity obtained, suggesting that the self-assembly of amelogenin M179 molecules to form stable and monodisperse aggregates requires the presence of the hydrophilic C-terminal sequence of M179.

Boyde: The work of Fearnhead and Höhling showed the self-assembly of longitudinal chains of spheres. They both had the notion that the mineral component was involved, although they couldn't possibly have interpreted this from their results because they were looking at heavy metal-stained material, and they were more likely to have stained protein aggregates (Fearnhead 1965).

Sasaki: You showed that there are crystal precipitates between amelogenin molecules but the orientation of apatite crystals is regulated by the cell. Those crystals are always perpendicular to the plasma membrane of the 'Tomes' process. Do you have any idea how amelogenin may regulate the orientation of apatite crystals?

Fincham: I agree that during the initial stages of development the crystallite is juxtapositioned to the cell membrane. I'm suggesting that the secretory ameloblast only sees the *c*-axial end of crystallite. The rest of the crystallite is encased in nanosphere structures, so that accretion of mineral ions on the developing crystallite,

coming through the ameloblast, occurs only at the *c*-axial end. The presumption is that the ameloblast is 'walking backwards, dragging a crystallite behind it'. We postulate that this is a dynamic situation, in that as more mineral is laid down so more amelogenin is laid down. Therefore, more nanosphere structures are formed and an aligned array of extremely thin early crystallites is produced.

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Tuftelin: enamel mineralization and amelogenesis imperfecta

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Abstract. Tuftelin is a novel acidic enamel protein thought to play a major role in enamel mineralization. Its identity and localization has been confirmed by amino acid composition, enzyme-linked immunosorbant assay, Western blots, indirect immunohistochemistry and high resolution protein-A gold immunocytochemistry. The deduced tuftelin protein (pI 5.2) contains 389 amino acids and has a calculated peptide molecular mass of 43 814 Da. Immunological studies suggest conservation of tuftelin structure between species throughout vertebrate evolution. The cDNA sequence encodes for several putative post-translation sites including one N-glycosylation consensus site, seven O-glycosylation sites and seven phosphorylation sites, as well as an EF-hand calcium-binding domain (with mismatch), localized towards the N-terminal region. At the C-terminal region (residues 252–345) tuftelin contains structurally relevant determinants for self assembly. We recently cloned and partially sequenced the human tuftelin gene (four exons have now been sequenced). These sequences include exon 1 and over 1000 bases of the putative promoter region. Employing fluorescent *in situ* hybridization, we mapped the human tuftelin gene to chromosome 1q 21-31. Localization of the human tuftelin gene to a well-defined cytogenetic region may be important in understanding the aetiology of autosomally inherited amelogenesis imperfecta, the most common enamel hereditary disease.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 135–155

Enamel, a unique and highly mineralized ectodermal tissue covering vertebrate teeth, is synthesized and secreted by specialized cells of the enamel organ called ameloblasts. The ameloblast cells and the secreted extracellular organic matrix (which are thought to be in constant communication) provide the ideal environment for the commencement of enamel mineralization and the growth of the elongated, thin mineral crystallites. As enamel further mineralizes and matures, most of the extracellular matrix proteins are degraded and replaced with the mineral ions calcium and phosphate, the enamel finally becoming the hardest and most mineralized (96% w/w) tissue in the body.

The extracellular organic matrix of enamel is composed of two major classes of proteins: the abundant (over 90%) hydrophobic proline-, histidine-, leucine- and

glutamine-rich amelogenins; and the acidic amelins, which are rich in aspartic acid, glutamic acid, serine and glycine (Termine et al 1980).

Amelogenin and amelins proteins have been postulated to play major roles in the structural organization and mineralization of developing enamel. Accumulating evidence and recent structural studies on recombinant mouse amelogenin have strongly suggested that the abundant hydrophobic amelogenins are involved in the control of mineral crystallite size, morphology and orientation (Fincham & Moradian-Oldak 1995). The acidic amelins, on the other hand, have been suggested by many researchers—on the basis of the timing of appearance (prior to the commencement of mineralization), localization at the dentine–enamel junction (DEJ), their secondary structure, their acidic nature and their binding to crystal surfaces—to be potential nucleators and regulators of enamel crystal growth (Termine et al 1980, Traub et al 1985, Slavkin et al 1988, Deutsch 1989).

Characterization of the acidic amelins

The amelins extracted from the developing extracellular matrix of enamel from a number of species have molecular weights of 70 kDa and below (Termine et al 1980, Deutsch 1989). Studies on the biosynthesis of enamel matrix proteins *in vivo* and *in vitro* have revealed the presence of four amelins species of approximately 70 kDa, 45 kDa, 30 kDa and 28 kDa (Ogata et al 1988). The existence of a number of amelins polypeptides with different molecular weights could be due to degradation of a high molecular weight amelins species, mRNA splicing or to the existence of a number of genes coding for different acidic proteins. In this context, it is interesting that cell-free translation studies employing the enamel organ mRNA of different species (human, bovine, porcine, mouse, rat and rabbit) indicated the presence of one or two nascent amelins proteins (Zeichner-David et al 1985).

Amino acid composition and 2D studies revealed the amelins protein to be acidic with a pI of 5.5 and below (Slavkin et al 1988, Deutsch 1989). The acidic nature of the amelins protein is in line with its ability to bind to the surface of the growing enamel mineral crystallites, as revealed by high resolution protein-A gold immunocytochemistry (Hayashi et al 1986).

Amelins have been reported to be glycosylated (Termine et al 1980, Menanteau et al 1988). Post-translational glycosylation of a protein will result in an increase in its molecular weight, compared to that predicted from its cDNA sequence or amino acid sequence obtained by Edman degradation.

A number of studies have suggested that the amelins proteins may be highly phosphorylated (for example Termine et al 1980). Identification of phosphorylation sites in the amelins proteins could be important in understanding the role of these proteins in enamel mineralization.

If amelins are to be involved in the initial mineralization of enamel, then they should be present at the very early stages of enamel formation (prior to mineral formation) and at the region of the DEJ, where mineralization commences. Indeed,

amino acid composition studies, high resolution 2D gel electrophoresis coupled with immunoblotting and immunocytochemistry, and cell-free translation assays have indicated that the first protein(s) to be expressed and secreted by the ameloblasts at the DEJ region are the enamelin(s) (compared to the amelogenins which are secreted only later) (Slavkin et al 1988, Deutsch 1989).

Another feature of enamelines, which might be important in understanding their role in mineralization, is associated with their secondary structure. X-ray studies (Traub et al 1985) suggested that the enamelines, which are rich in aspartic acid, could be arranged in a β -sheet conformation, and that functional amino acid groups (e.g. aspartic acid) could nucleate the mineral component. Some of the β -sheet enamelin proteins that Traub et al (1985) studied were preferentially orientated perpendicular to the crystallographic c -axis of the mineral, implying a specific role of these proteins in controlling crystal nucleation and growth.

The enamelin proteins initially secreted at the very early stages of enamel development persist throughout the forming and maturing stages of development and are present in the mature tissue (some in a partially degraded form) as part of the mature enamel proteins referred to as tuft proteins (Deutsch et al 1991a,b, 1995). Indirect immunohistochemical studies employing polyclonal antibodies against tuft proteins have shown that the origins of some of the tuft proteins are the acidic 50–70 kDa enamelin proteins secreted during early stages of enamel formation (Robinson et al 1989).

Indirect immunohistochemistry and high resolution protein-A gold immunocytochemistry have indicated that the acidic enamelin proteins have common antigenic determinants across a wide range of vertebrates, including shark and fish, suggesting that at least portions of the enamelin protein structure have been highly conserved throughout 450 million years of vertebrate evolution (Slavkin et al 1984) and that these acidic extracellular enamel matrix proteins have an important biological role in the process of enamel mineralization.

In recent years it has become clear that some of the proteins found in what was originally described as the enamelin-enriched fraction (see Termine et al 1980) are serum albumin proteins (Strawich & Glimcher 1990). However, it has now been shown unequivocally that serum albumin is not expressed and synthesized by the ameloblast cells in normal enamel. The presence of serum albumin in developing enamel is probably due to contamination during tissue preparation. Northern and Southern blot analyses by Couwenhoven et al (1989) and more sensitive PCR studies by Yuan et al (1996) failed to show any expression of serum albumin in ameloblasts. In addition, the recent studies of Chen et al (1995) showed that if the developing tissue was first freeze dried and then extracted, no serum albumin could be detected in developing enamel. This finding does not exclude the possibility of the *in vivo* presence of serum albumin in pathological forming enamel.

The underlying desire to find new proteins that are secreted by the ameloblast cells, that are specific to the developing enamel tissue and that may have a major role in enamel mineralization has recently resulted in the characterization of new proteins.

The major acidic enamel proteins, which have now been characterized and at least some of which belong to the group of proteins originally described in the past as the enamelin proteins, are: (1) the acidic tuftelins, originally cloned and sequenced (bovine and human) by Deutsch et al (1991a,b, 1994, 1995), and more recently (murine) by Zeichner-David et al (1995), Simmons et al (1996) and Ware & Dickwisch (1996); (2) the acidic sulfated proteins (up to 65 kDa), which have a rapid turnover (recently identified by Smith et al 1995); and (3) the novel acidic (proline-rich) enamel protein recently discovered in the rat and mouse simultaneously and independently by two different laboratories, which called it either ameloblastin (Krebsback et al 1996) or amelin (Fong et al 1996). Simmer et al (1996) have now cloned the porcine form of this protein. The ameloblastin/amelin protein was reported to contain an integrine recognition sequence (Fong et al 1996), thus indicating its possible role in the communication between the ameloblast cell and the enamel extracellular matrix. A fourth protein, which was originally described as a proline-rich, non-amelogenin protein and later as a 32 kDa enamelin protein (the precursor of which was thought to be the 89 kDa enamelin), and which is thought to play a role in enamel mineralization, has now been cloned and cDNA sequenced by Fukae et al (1996). Moradian-Oldak et al (1996) have shown that this 32 kDa protein is a protease that degrades specifically the amelogenin protein and that the 89 kDa protein is its precursor.

Primary structure and localization of tuftelin

Despite the many studies on enamelin proteins, which have been described following the isolation of enamelin by Termine et al (1980), virtually nothing was known about the enamelin primary structure. No amino acid sequence typical of enamelin had been reported, and neither had any gene for these proteins been identified. Deciphering the enamelin primary structure was crucial because many researchers considered these acidic enamel proteins to be potential nucleators and regulators of enamel crystal growth. It was clear that the elucidation of even a short unique sequence would provide the identity card for what seemed to be an important protein in enamel mineralization.

We have now cloned, cDNA sequenced and characterized a novel acidic enamel protein (which we called tuftelin) belonging to the family of proteins originally classified as enamelins (Deutsch et al 1991a,b, 1994, 1995). The identity, expression and ultrastructural localization of tuftelin was confirmed by amino acid composition, indirect immunohistochemistry, Western blot analysis and high resolution protein-A gold immunohistochemistry employing synthetic tuftelin peptide antisera (Deutsch et al 1991a,b, 1995).

The deduced protein is hydrophilic (see Fig. 1), acidic, and rich in glutamic and aspartic acid (see Fig. 2). It contains 389 amino acids (Fig. 3) and has a calculated peptide molecular mass of 43 814 Da.

Post-translational modification of the nascent tuftelin protein, such as glycosylation, would increase the molecular weight predicted from its amino acid or cDNA sequence.

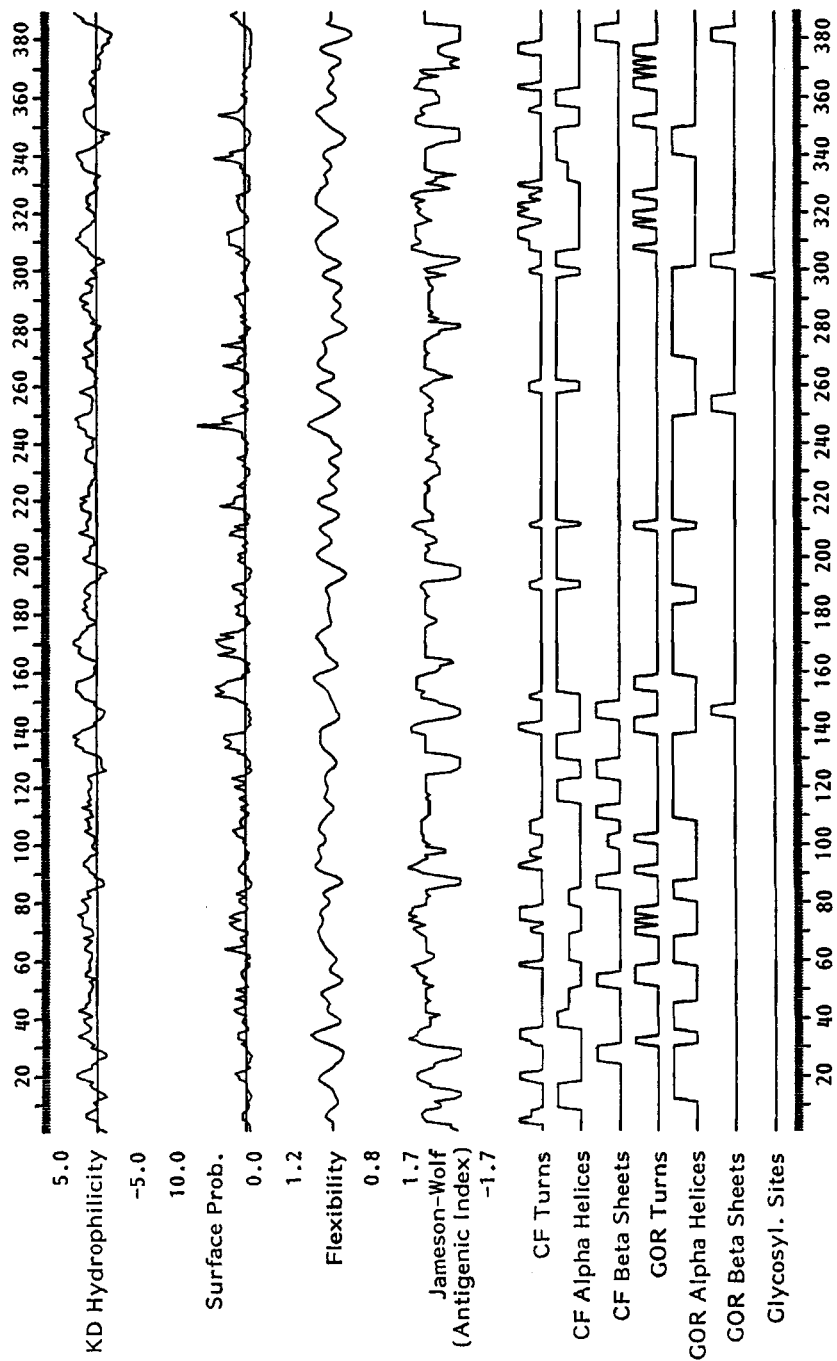


FIG. 1. Plot structure (GCG computer program) of the deduced tuftelin protein sequence (Deutsch et al 1991a). The graph shows the hydrophilicity (K_D), surface prob., flexibility and Jameson-Wolf antigenic index of tuftelin. The predicted Chou-Fasman (CF) and G. O. Robinson (GOR) predicted β -sheets, α -helices, turns and N-glycosylation sites are also shown.

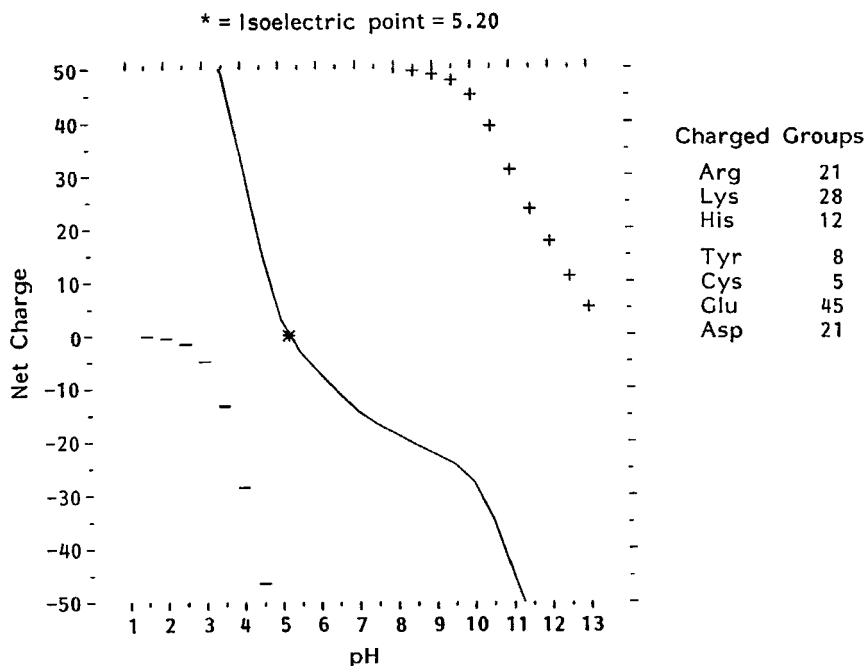


FIG. 2. The isoelectric point of the deduced tuftelin protein sequence (Deutsch et al 1991a) as determined by the GCG computer program.

One potential N-glycosylation site is at residue 298 (see Fig. 3). In addition, seven potential O-linked glycosylation sites have been predicted according to the method of Hansen et al (1995). These include three threonine O-glycosylation sites (at residues 7, 102 and 304) and four serine O-glycosylation sites (at residues 6, 104, 105 and 362). Western blotting of an enamel-enriched fraction with synthetic tuftelin peptide antisera (Deutsch et al 1991a,b, 1995) reveal the presence of enamelin with molecular masses of 66–58 kDa, about 48 kDa and 28 kDa (and below). These are typically recognized by affinity purified polyclonal antibodies (Deutsch 1989) and have been shown to be synthesized by the ameloblast cells (Ogata et al 1988).

Phosphorylation of the tuftelin protein could provide sites for the specific chelation of calcium ions, and thus could be important in understanding the role of these proteins in enamel mineralization. Seven serine and threonine phosphorylation consensus sites have now been identified. Five of these have casein kinase II (CK2) recognition sequences, two of which have serine phosphorylation sites (SKLD and SPPE at residues 70 and 105, respectively), and three of which have threonine phosphorylation sites (TWQD, TALE and TIQE at residues 123, 203 and 270, respectively). The remaining two have protein kinase C consensus sequences: SSK and SLR at residues 69 and 119, respectively (see Fig. 3).

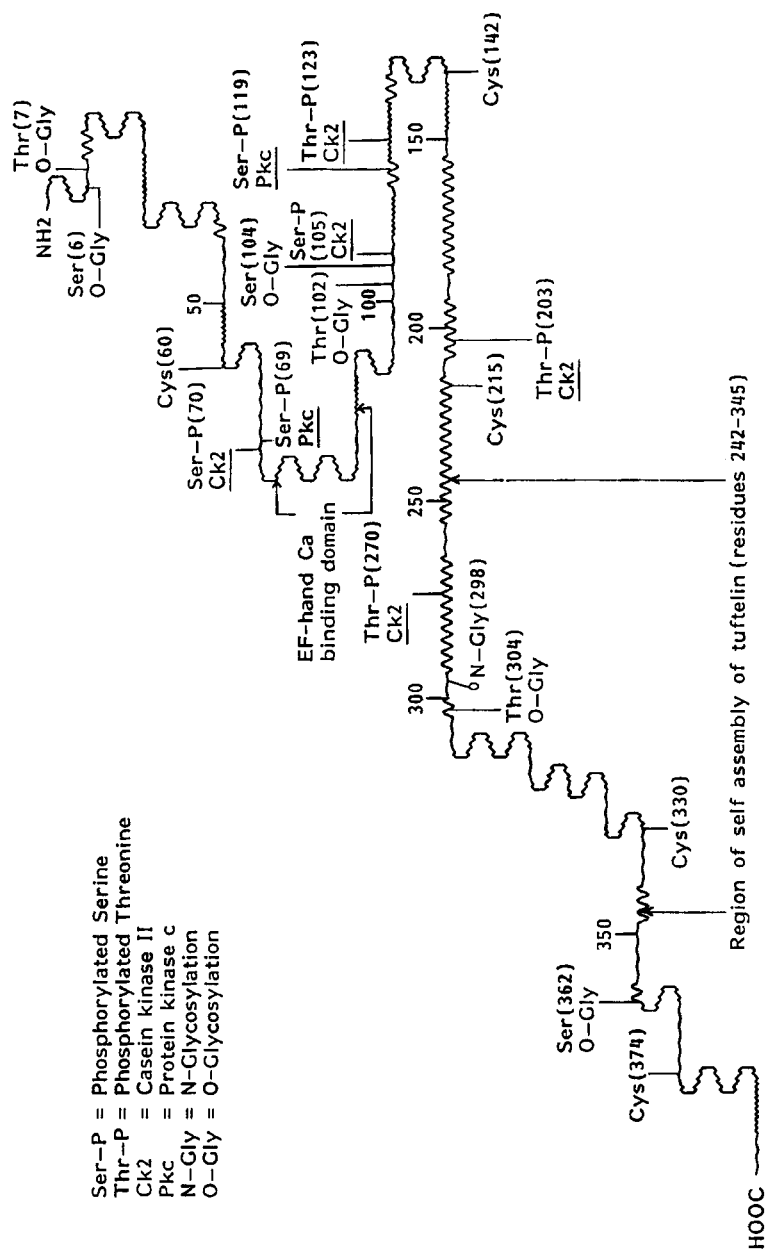


FIG. 3. The positions of the deduced tuftelin self-assembly region (Paine et al 1996), the EF-hand calcium-binding domain consensus site, phosphorylation and N-glycosylation consensus sites, and predicted O-glycosylation sites superimposed on the predicted Chou-Fasman model for protein secondary structure.

Five cysteines have been identified (residues 60, 142, 215, 330 and 374). The presence of cysteines might suggest the existence of (up to two) disulfide bonds, which are generally important in stabilizing the structure of extracellular proteins.

One interesting consensus sequence (with mismatch) found in tuftelin is the EF-hand calcium-binding domain. This consensus sequence has been identified in about 30 proteins, some of which are intracellular but at least one of which is an extracellular secreted protein, osteonectin, which is a non-collagenous bone protein. A mismatch(s) in the relatively large consensus sequence might indicate a weaker association with calcium ions (normally present at this site in the other calcium-binding proteins). This weaker association in turn might be an important characteristic of a possible nucleating protein. The EF-hand consensus sequence was located towards the N-terminal region of the tuftelin protein (DRDPGDSVHKQEI, residues 73–85). In this sense, it is interesting that Paine et al (1996), employing the yeast two-hybrid system, showed that tuftelin could self-assemble and that tuftelin amino acid residues 252–345 (adjacent to the C-terminal region) contain structurally relevant determinants for tuftelin self-assembly. Perhaps this special structural arrangement of a self-assembly C-terminal region, with a non-self-assembly N-terminal region, containing a calcium-binding domain and many phosphorylation consensus sites and predicted O-glycosylation sites, is a structural motif important in the role of tuftelin in enamel mineralization. Future structural studies (e.g. crystallization and X-ray diffraction) will establish which of the predicted and consensus sequences are real.

The tuftelin sequence contains the peptide sequence -Pro-Ser-Pro-Pro-Ala-Gln-. A homologous sequence, -Pro-Ser-Ser-X-X-Ala-Gln-, is found in an N-terminal decapeptide sequence of a 22 kDa unidentified protein band isolated from an enamel extract (Strawich & Glimcher 1990) (the latter sequence has an extra serine). This may indicate that the published peptide sequence (Strawich & Glimcher 1990) is a tuftelin derivative or fragment. Attempts are currently being made to overexpress tuftelin in *Escherichia coli* (Deutsch et al 1995) and baculovirus systems to further characterize tuftelin structure and function.

Indirect immunohistochemical and high resolution protein-A gold immunocytochemistry studies at the light and electron microscope levels, respectively, using synthetic tuftelin peptide antisera (Deutsch et al 1991, 1995) showed that the antibodies react with the forming ameloblast cells, particularly with the secretory region (Tomes' process), and with the underlying developing extracellular enamel matrix. No apparent reaction is observed with either the odontoblast (dentine-secreting cells) or the extracellular dentine matrix. The antisera also reacted with bovine and human mature enamel tuft proteins, radiating from the DEJ towards the enamel surface. This suggested that at least some of the tuftelin, which is secreted during the early stages of enamel formation, remains throughout the development of enamel and is found (perhaps in a partially degraded form) in the mature tissue as part of the tuft proteins.

High resolution protein-A gold immunohistochemical studies (Deutsch et al 1995) confirmed that the tuftelin acidic enamel protein is localized in the ameloblast cell and in extracellular enamel matrix. Virtually no tuftelin is found in the odontoblast or in the

extracellular dentine matrix. The results further showed that in the ameloblast tuftelin is localized in the rough endoplasmic reticulum and in vesicles and secretory granules concentrated at the Tomes' process. Tuftelin is present in the extracellular matrix, mainly at the immediate crystallite regions, confirming earlier results on the ultrastructural localization of enamelin. Its distribution throughout the thickness of enamel is not, however, homogeneous. Tuftelin, it seems, is more concentrated at the DEJ (see also biochemical and indirect immunohistochemistry results of Termine et al [1980] and Deutsch [1989], respectively, and recent results on tuftelin distribution in enamel by Ware & Diekwisch [1996]). Preliminary studies employing double-labelling high resolution protein-A gold immunocytochemistry indicate that tuftelin and amelogenin proteins do not co-localize in the cell and are found in different vesicles and secretory granules (Deutsch et al 1995).

Immunological studies reveal cross-reactivity between tuftelin of different species—such as human, bovine, rat, rabbit and shark—suggesting high conservation of tuftelin structure between species and throughout vertebrate evolution (Deutsch et al 1991a, 1995). Vahadi et al (1996) have shown that anti-tuftelin antiserum also reacts with the outer layer of the pharyngeal teeth of the Pacific hagfish (*Eptatretus stoutii*, an even more ancient vertebrate species). The conservation of tuftelin throughout millions of years of evolution suggests that it has an important biological role in the development and mineralization of enamel.

Using our bovine tuftelin cDNA data (Deutsch et al 1991a), Zeichner-David et al (1993) have produced specific tuftelin oligonucleotide probes, and using Northern blot hybridization they have showed that tuftelin is also expressed in mouse ameloblasts from early enamel formation to maturation. It is not expressed in mesenchymal odontoblast cells. Recently, they partially sequenced a murine tuftelin cDNA clone and showed that it has a high degree of homology with the bovine tuftelin cDNA (Dodds et al 1996). In addition, using a synthetic peptide antiserum made to our predicted bovine tuftelin sequence (Deutsch et al 1991a) and indirect immunohistochemistry, Zeichner-David et al (1995) showed that tuftelin is first secreted at very early stages of enamel development, before the bulk of amelogenin is secreted. Zeichner-David et al (1995) placed the initial expression of tuftelin at the bud stage. Simmons et al (1996) have reported that tuftelin is the first protein (amongst the known enamel proteins) to be expressed by the ameloblast. This is followed by the expression of ameloblastin/amelin, and then the expression of amelogenin.

The role of tuftelin/enamelin in enamel mineralization

The acidic nature, some aspects of its 2D structure, its binding to the crystal surface and the timing of its secretion (which commences prior to enamel mineralization) have suggested to many researchers that tuftelin/enamelin protein(s) might be a potential nucleator and regulator of enamel crystal growth.

The presence of a relatively high concentration of tuftelin at the DEJ during early stages of development has suggested to some that tuftelin/enamelin might be involved

with the mineralization of the hypermineralized enamel region adjacent to the DEJ (present in the very early-forming enamel of all species) (Fearnhead 1979), thereby creating a mineralization front in enamel (Deutsch et al 1991a, 1995). This idea implies that the ameloblast cells, together with the early-forming extracellular enamel matrix which they secrete at the region of the DEJ, could provide their own micro-environment for the commencement of initial enamel mineralization (nucleation and growth of thin enamel crystallites). Arsenault & Robinson (1989) suggested, amongst others, that the initial mineralization of enamel also involves the matrix or minerals of the underlying dentine, that the acidic tuftelin/enamelin might bind to the collagen fibre of the underlying dentine, and that such a chemical interaction between the two matrices may promote enamel crystal nucleation.

MacDougall et al (1994) have reported, however, that ameloblast cells *in vitro* can synthesize and secrete an enamel extracellular matrix which is capable of *de novo* biomineralization in the absence of any pre-existing dentine matrix and crystals. In addition, Zeichner-David et al (1995) have reported that the first enamel crystallites formed are not found in continuity with dentine components.

Since tuftelin has been shown to be expressed at very early stages of amelogenesis (pre-secretory stages), prior to the commencement of enamel mineralization, it may have other roles in the initial mesenchyme-ectoderm interaction and communication leading to enamel and dentine formation.

The human tuftelin gene and its chromosomal localization

We have recently cloned (from a human genomic library contained in Strategene Lambda Fix) and partially sequenced (four exons have now been sequenced and a fifth exon identified) the human tuftelin gene (Deutsch et al 1994), employing the bovine tuftelin cDNA probe (Deutsch et al 1991a). These sequences included exon 1 and over 1000 bases of the putative promoter region. Southern hybridization and sequencing indicated that the clone probably contains the entire gene. Employing fluorescence *in situ* hybridization, we mapped the human tuftelin gene to chromosome 1q21-31 (Deutsch et al 1994). The mapping of the human tuftelin gene to a well-defined cytogenetic region could be crucial in understanding the aetiology of autosomally inherited amelogenesis imperfecta, the most common hereditary disease of enamel.

The human tuftelin gene and amelogenesis imperfecta

Amelogenesis imperfecta is a heterogeneous group of hereditary disorders that affects primarily the enamel of teeth. The different types of amelogenesis imperfecta have been subclassified according to their clinical appearance, enamel characteristics and mode of Mendelian inheritance. The enamel abnormalities have been categorized as hypoplastic, hypocalcified, hypomaturational or a combination of these. The inheritance patterns include autosomal dominant, autosomal recessive, X-linked

dominant or X-linked recessive modes of transmission. Having mapped the amelogenin gene to the X and Y chromosomes, it was first suggested by Lau et al (1989) and then confirmed by Lagerström et al (1991), Lagerström-Fermér et al (1995) and Aldred et al (1992), for example, that mutations of the gene encoding this most abundant enamel protein are associated with the X-linked amelogenesis imperfecta.

Inherited X-linked amelogenesis imperfecta, however, comprises only a small fraction of all amelogenesis imperfecta cases. A large proportion of amelogenesis imperfecta cases are autosomally linked and could therefore only be explained by mutation of genes residing on autosomal chromosomes. A mutated form of the acidic tuftelin is a good candidate for the cause of at least one form of autosomal amelogenesis imperfecta because it is specifically expressed in the ameloblast, it is secreted in the early stages of amelogenesis, it persists throughout the development and mineralization of enamel, it is conserved throughout vertebrate evolution (Deutsch et al 1991b) and its gene is localized to an autosomal chromosome (chromosome 1). Attempts are currently being made to study the involvement of the human tuftelin gene in more than 20 families with autosomally linked amelogenesis imperfecta (one or more families for each of the available subclasses of this hereditary disease have been identified and characterized, and DNA samples from affected and non-affected members will be available for this study). Bäckman et al (1995) reported a linkage between autosomally inherited amelogenesis imperfecta and chromosome 4. In this respect, it is interesting that ameloblastin (Krebsbach et al 1996) localizes in the mouse to chromosome 5 (the equivalent to chromosome 4 in humans). This accumulating information will provide a baseline for further understanding the molecular mechanisms involved in these hereditary diseases.

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DISCUSSION

Sasaki: Is tuftelin expressed before amelogenin in ameloblasts?

Deutsch: We have done some preliminary *in situ* hybridizations in which we have observed that tuftelin is expressed in ameloblasts prior to amelogenin. As enamel mineralization commences, the expression of tuftelin increases in the ameloblasts. In the continuously growing rat incisor, tuftelin is expressed in pre-ameloblasts but only in the labial side and not in the lingual side, the latter containing only dentine but not enamel. We have also used antibodies against tuftelin peptides which have demonstrated that some of the protein is localized in secretory granules.

Sasaki: Do the enamelins accumulate in the basement membranes?

Deutsch: From indirect immunohistochemistry it seems that enamelins accumulate in the vicinity of the basement membranes.

Zeichner-David: We'll have to revise our prior statements as more sensitive techniques to determine gene expression are developed. For example, the expression patterns of amelogenin and tuftelin depend on the technique used. Using reverse transcriptase (RT) PCR methods, expression of tuftelin cDNA in the embryonic mouse is first detected at embryonic day (E) 13. However, the protein doesn't

accumulate until E17, when it can be detected by immunohistochemistry. Similarly, we used to believe that amelogenin was present only in new-born mice because that's when the antibodies detected it, but RT-PCR analysis has demonstrated that the transcripts are present at E15. In my opinion, it is also necessary to do this sort of revision with the expression of dentine proteins.

Boydé: I would like to question the name tuftelin, on the grounds that tufts are equivalent to geological faulting planes and develop in the early maturation stage probably because of internal pressures. The faulting planes are filled in by enamel matrix proteins from the surrounding enamel. Therefore, the word 'tuft' could be replaced by something meaning 'garbage collection zone'.

Mann: If tuftelin is expressed and secreted early on, so that it's present at the dentine-enamel junction (DEJ), is there any evidence that it binds to collagen at the interface?

Deutsch: Arsenaault & Robinson (1989) have suggested that this might occur.

Zeichner-David: We have found that tuftelin is localized at the DEJ junction, but it continues to be expressed during the maturation stage, suggesting that it may be important for crystal nucleation (Zeichner-David et al 1995). However, it is present in only very low amounts; i.e. about 1000-fold less than ameloblastin.

Yamada: I would like to clarify that although ameloblastin is distinct from amelotin, they are two different proteins encoded by two different genes, although, apparently, amelotin and the sheath protein are identical to ameloblastin. My question concerns the ability of tuftelin to aggregate; that is, can it also interact with amelogenin?

Snead: We have used a genetic approach, called the yeast two-hybrid system, to address this (Fig. 1). This method can identify the capacity of two proteins to interact by their ability to reconstitute the GAL4 transcription apparatus. GAL4 can be separated into two domains: the DNA-binding domain and the activating domain. If the GAL4 transcription apparatus is separated into these two pieces, and if: (1) another gene—such as amelogenin, tuftelin or ameloblastin—is cloned into each of these GAL4 pieces; and (2) the proteins interact with each other, then the GAL4 transcription apparatus is re-constituted and a blue colour is observed. This is a quick way of screening for protein-protein interactions.

Using this system, we had hoped to find domains within amelogenin responsible for the assembly of the enamel matrix that segregated along exon boundaries. We found that although the interaction domains come close to exon boundaries, they do not perfectly segregate with them. We identified two domains: (1) the highly conserved first 42 amino acid stretch at the N-terminus of amelogenin, which we call domain 'A'; and (2) a domain at the C-terminus, excluding the carboxyl peptide hydrophilic terminus that we call domain 'B', which accounts for the self-assembly properties of amelogenin. The amelogenins do not interact if either domain is deleted.

Tuftelin also has self-assembly properties, which have been localized to a single domain located at the C-terminus of tuftelin including amino acid residues 272 through 345 (Paine et al 1996). Thus, tuftelin appears to segregate into a self-assembly motif at the C-terminus and a calcium-binding motif, characterized by the high phosphoserine groups at the N-terminus. The amelogenins and tuftelins do not

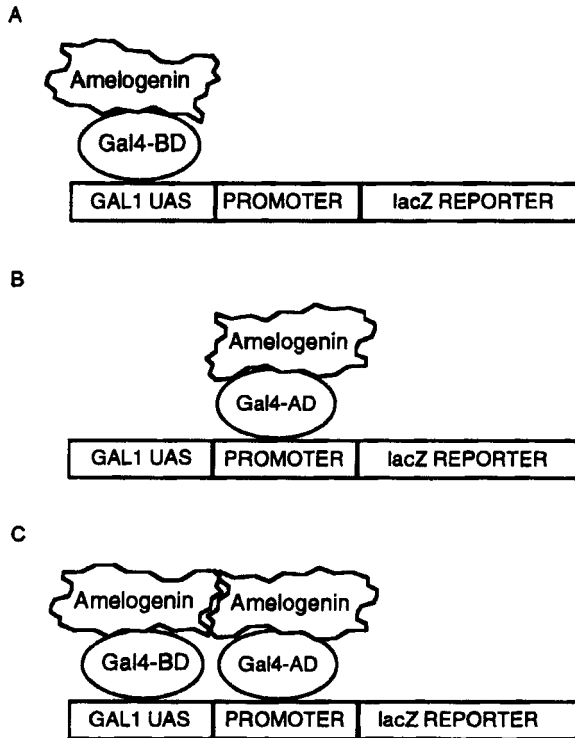


FIG. 1. The yeast two-hybrid system. (A) A fusion protein formed from the GAL4 DNA-binding domain (BD) and amelogenin binds to the GAL1 upstream activating sequence (UAS) but cannot activate reporter transcription in the absence of the activation domain. (B) A fusion protein formed from the GAL4 activation domain (AD) and amelogenin binds but cannot independently activate transcription of the reporter without the binding domain. (C) Interaction between the two amelogenin fusion proteins reconstitutes GAL4 function and results in the transcription of the *lacZ* reporter gene, roughly proportional to the avidity of the amelogenin-amelogenin interaction.

interact with one another in the yeast two-hybrid system, suggesting a two-phase enamel extracellular matrix in which the amelogenins and tuftelins self-assemble separately from one another. We are now using the yeast two-hybrid system to screen for proteins that may bridge the interaction between amelogenins and tuftelins in the extracellular matrix. Michael Paine, Yoshi Yamada and Paul Krebsbach have shown that ameloblastin does not appear to self-assemble (unpublished results 1996). At this time then, two out of the three cloned enamel proteins are capable of self-assembly. The full-length amelogenins produce self-assembled structures, but progressive deletions at the C-terminus result in a diminished capacity of these molecules to self-assemble. This is also in keeping with

the increase in LRAP (leucine-rich amelogenin protein) concentration observed during the maturation phase of enamel. Therefore, it appears that progressive C-terminal cleavage results in a reduction in amelogenin self-assembly, which may allow the increased expansion of the enamel crystallites as the nanosphere assemblies become smaller.

Fincham: We have some results which demonstrate that it is difficult to obtain monomeric amelogenin. It is assembled in some way into 4–5 nm structures, containing of the order of four, five or six molecules. Is it possible to use the two-hybrid system to explain the formation of these structures which form a chain of molecules with recognition sites that link one end of a molecule to another?

Snead: I can't answer that because of the limitations of the two-hybrid system.

Fincham: The problem is that in order for these molecules to link up they have to have a 'hook and an eye'.

Snead: The two-hybrid system can define the hook and the eye, but it cannot demonstrate conclusively that the hook and eye lead to assembly during enamel formation *in vivo*. We have found the hook and the eye, so now we're using other techniques, such as transgenic animals, to introduce defects in the assembly domain of amelogenin.

Yamada: I have a technical question. Did you check the expression levels of the recombinant protein during this experiment?

Snead: This is a good point. Yoshi Yamada is asking whether a reported negative interaction may be due to the protein being degraded prior to being able to interact. We have shown using Western blot analysis that in all cases the protein is present, even when the protein is truncated. We have also shown that the phenotype can be rescued by expressing protein in which the deletion has not occurred.

Simmer: The open reading frame of the original tuftelin clone continued on past the 5' end of the cDNA and there may have been an ATG start codon more 5' which would not have been translated into a protein with a signal peptide. This leads me to conclude that there is a good possibility that the original clone was abbreviated and that there is more (unknown) sequence at the 5' end of the tuftelin mRNA. Has the characterization of the bovine tuftelin genomic sequence demonstrated a promoter region upstream of the sequence that corresponds to the 5' end of the tuftelin cDNA?

Rosenbloom: No. We've identified 13 exons and, as you point out, an open reading frame extends 5' of the ATG designated as the initiation methionine in the published sequence now included in exon 1. However, when we performed primary extension experiments, the results were equivocal because the tuftelin mRNA levels were low. We've tried using a region upstream of exon 1, but it does not have the characteristics of a promoter. There is probably another either untranslated or translated exon further upstream that we have not yet identified.

Snead: One of the ways of changing the stoichiometry of the extracellular matrix, in terms of its protein components, is by changing the alternative splicing that occurs, and thereby changing the mRNA species that can be translated. Could you describe your results regarding alternative tuftelin splicing in relation to our results, which

suggest that on one side of the tuftelin molecule there is an assembly domain whereas the other part contains a calcium-binding domain? What happens to the spliced isoforms of bovine tuftelin?

Rosenbloom: The alternatively spliced forms that we've identified so far vary only in the N-terminal region of the two protein forms, so tuftelin does not have the same degree of alternative splicing that is observed in the amelogenins.

Snead: So it would be safe to conclude that the self-assembly domain is intact in the different isoforms?

Rosenbloom: Yes, I believe so. I also have a question for you. You mentioned LRAP, and that the N-terminal and C-terminal portions of amelogenin are the most important regions for self-assembly, but that is exactly what LRAP is, so wouldn't one predict that it would be able to self-assemble?

Snead: Firstly, I would like to mention that the two-hybrid system is not a perfect system and the fine mapping of amino acid residues contributing to a self-assembly domain is a currently being performed. In this regard, Michael Paine, who has carried out these studies, must be congratulated for his hard work. However, we now have some idea of where the 'B' domain terminates, i.e. at around residue 157. We have also found that LRAP will interact with full-length amelogenin, but only weakly and not with itself. Weak assembly may result from the need to have some amount of spacing between the two domains, so that the proper orientation of the molecule is maintained. Tuftelin interaction with itself is about 10 times stronger than that between p53 and large T antigen, which is taken among investigators in nuclear transcription as a strong interaction between proteins. However, the amelogenin self-assembly interaction is about twice as strong than that between p53 and large T antigen.

Veis: Is an ordered array formed during the self-assembly interaction?

Snead: The two-hybrid system cannot answer this question.

Fincham: We now have a synthetic LRAP, although we haven't yet had the chance to look at the possible self-assembly properties of this molecule.

I would like to discuss the definitions of 'amelogenin' and 'enamelin'. The term 'enamelin' came into common use in 1980 when John Termine introduced a sequential extraction procedure to isolate developing enamel proteins (Termine et al 1980). This procedure involves first an extraction with 4M guanidine HCL buffered at pH 7.4, and then an extraction with guanidine containing EDTA. In the original paper the first extract contained what was called 'amelogenin', and the second extract contained what was called 'enamelin' (A. G. Fincham, unpublished results 1985; see Fig. 2). We took 8.2 g moist weight of bovine enamel matrix protein and subjected it to the sequential extraction procedure, except that we carried out five sequential extractions in guanidine and three in guanidine plus EDTA. We observed that almost all the protein (97%) was present in the first extract (the so-called 'amelogenin fraction'). There was another, much smaller peak in the first EDTA extraction (1.7% of the total protein), which represents the protein released as a result of demineralization (the so-called 'enamelin fraction'). The overall recovery in this experiment was

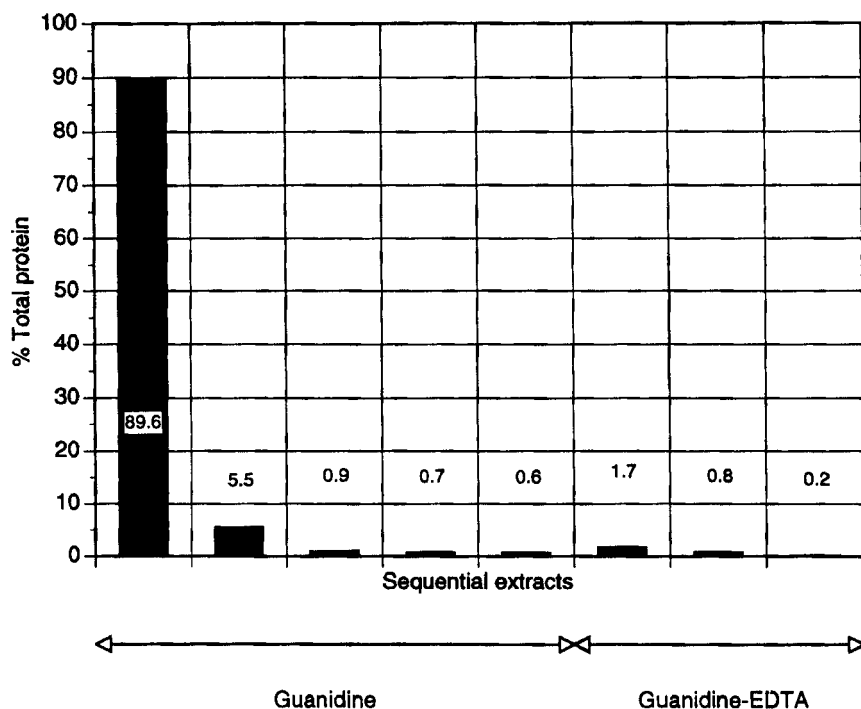


FIG. 2. Sequential extraction of bovine enamel matrix proteins. Bovine enamel matrix (8.2 g moist weight) was scraped from developing bovine molar teeth. The scrapings were extracted sequentially with 6M guanidine hydrochloride pH 7.4 (extracts 1–5) and then with 6M guanidine hydrochloride pH 7.4 containing 0.5 M EDTA (extracts 6–8) (procedure of Termine et al 1980). The estimated overall recovery of protein was 67–70%.

estimated as about 67–70%. However, gel electrophoresis showed that the first extract contains proteins of a higher molecular mass than any known amelogenin, and similarly, that there are proteins in the second ('enamelin') extract that have the same molecular mass as amelogenins. Therefore, a more precise and detailed terminology, which is not based on methodology, is required.

Slavkin: As a footnote, the Termine et al (1980) paper was derived from the enamel meeting held at Dulles Airport near Washington, DC in 1979. When John Termine was preparing his presentation, he became challenged by the complexity of enamel, so he sat down to discuss this with his colleague Vincent Hasscal, at the National Institute of Dental Research, National Institutes of Health. Vince had been using sequential guanidine extractions, with and without EDTA, to fractionate the proteoglycans from cartilage. John borrowed that technology from Vince, which illustrates the

benefit of having talented people from different disciplines looking at the same problem.

Veis: Another important aspect that we should not forget is that the first extract contains non-ameloblast proteins, such as serum proteins, which may have a role in crystal formation because they're present when the initial crystallization is occurring.

Robinson: The serum albumin present during secretion does not seem to bind to the crystals *in vivo*, although it has the capacity to do so. This suggests that the enamel matrix may have another function, i.e. to protect the growing crystals from potentially inhibitory molecules such as albumin. During maturation, however, when the amelogenin matrix has been largely degraded and removed, albumin can become attached to the exposed crystals and is in turn degraded by the scavenging serine protease system present at that stage. If albumin enters enamel the wrong time or if it overwhelms the local serine protease scavenging system then a problem is created. I will address this further in my chapter (Robinson et al 1997, this volume).

Slavkin: And Tom Diekwisch has observed that crystallites can form many micrometres away from the plasma membrane, suggesting that there are also molecular interactions independent of the plasma membrane where nucleation and early crystal growth take place.

Nanci: Slavkin et al (1988) clearly demonstrated that enamel proteins are expressed before birth. We have further shown that proteins immunoreactive with an antibody to mouse amelogenin initially associate with filaments of the basement membrane separating differentiating ameloblasts and odontoblasts (Nanci et al 1989), and more recently, we have hypothesized that some of the first enamel proteins may interact with fibronectin during early odontogenesis (Sawada & Nanci 1995). In some recent unpublished immunocytochemical work with Yoshi Yamada's group, we obtained a similar result with ameloblastin that is also secreted early during the pre-secretory stage. Since mineralized dentine is not present at that time, early ameloblast products can diffuse into the forming mantle dentine. Nakamura et al (1994) reported the diffusion of amelogenin along odontoblast processes and its uptake by these cells. Indeed, the diffusion of resorcin-fuchsin stainable ameloblast products deep into dentine and pulp has been shown by Suga (1960).

Zeichner-David: Is enamel present?

Nanci: At the time when extracellular amelogenin and ameloblastin are first immunodetected there is no mineralized layer of dentine or enamel. The first proteins are somewhat dispersed throughout the forming extracellular matrix in adjacent ameloblasts, and they then accumulate as patches that show no morphological evidence of crystal formation.

Slavkin: Using immunocytochemistry, several research groups, including our group in Los Angeles, discovered that enamel protein antigens, cross-reactive and produced against specific synthetic amelogenin polypeptide immunogens, were localized not only along the traditional intracellular secretory pathway of inner enamel epithelial cells (Kallenback zone 4 epithelia) but were also localized in the forming enamel matrix and along the surfaces of extended odontoblastic cell processes. The

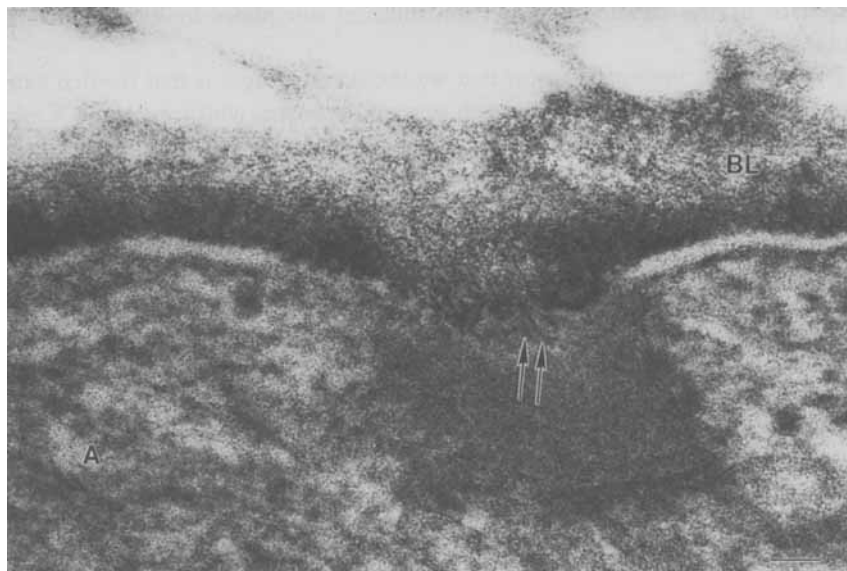


FIG. 3. In hornsharks, initial enameloid mineral (arrows) is formed along the thick basement lamina (BL) and the ameloblast (A) cell membrane. Bar = 50 nm.

amelogenin antigens were also localized in lysosomes within pre-odontoblast cells. This represented the possible transmission of molecular 'information' or signal from inner enamel epithelia to adjacent dental papilla mesenchymal cells. Bill Butler has presented the localization of dentine sialoprotein antigens within inner enamel epithelia as well as within dental mesenchyme (Butler et al 1997, this volume). We also see from Danny Deutsch the secretion of collagen and enamel antigens from inner enamel epithelia in shark tooth development. From my perspective, these results suggest that there may well be intercellular molecular communication and the precipitation of a collection of these molecules along the putative DEJ. How these various molecules interact with one another is the newest challenge and Mal Snead's approach using the two-hybrid system seems promising. The next generation of structural biology studies of enamel matrix molecular interactions should be exciting.

Snead: This may be too much of a reductionist review, but one more piece of that puzzle comes from your own observations on amelogenin in the hagfish and the analogy of compartmental segregation during enamel formation. The two-hybrid system has demonstrated that tuftelin and amelogenin do not interact. I did not expect this to be the case; therefore, it struck me that if they do not interact *in vivo* they must have certain limitations imposed on them, such as being contained within separate compartments. Alternative splicing of amelogenin isoforms in the mammalian model may lead to regulated assembly of the matrix, but in the shark and

hagfish the same proteins are shuffled together and mixed with collagen. How can these enamel proteins have such different assembly properties? There should be some commonality of development and phenotype.

Diekwisch: This could be answered if we look at the question from an evolutionary point of view. Mammalian enamel probably evolved relatively recently. Higher vertebrate amelogenins are probably highly unique, specialized proteins that form a matrix to allow the formation of long, parallel crystals. Mammalian enamel, however, is the end product of a heterochronic process, and it contains features from earlier stages of enamel evolution. In sharks and rays mineral clusters are formed at interfaces between different kinds of proteins. In these species we have observed sites of initial mineral formation at the cell membrane and ameloblast basement membrane, as well as in the interface between mantle dentine and enamel matrix (Fig. 3). The concept of minerals forming between protein aggregates is probably conserved during evolution but the formation of long, parallel crystals by specifically aggregated amelogenins has evolved only relatively recently in higher vertebrate enamel.

Snead: What are the intermediate species?

Boyde: Reptiles. They do not have Tomes' processes and they have long thin crystallites that are perpendicular to the surface of the enamel.

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Enamel maturation

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Abstract. Enamel maturation is characterized by massive crystal growth in both width and thickness, resulting in the most highly mineralized of all mammalian skeletal tissues. The control of this process is mediated via a carefully orchestrated series of events that are temporally and spatially regulated, and it requires the co-ordinated degradation and removal of the endogenous enamel matrix. This is affected by both neutral metalloproteases and serine proteases, which are developmentally restricted and may be further modulated by changes in the chemistry of the enamel crystals themselves. Failure of these mechanisms, or the adventitious entry of mineral-binding proteins during the later stages of maturation, may result in the incomplete maturation of the enamel crystals and the eruption of dysplastic tissue.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 156–174

The stages of enamel development

Fully mature enamel is acellular and comprises 80–90% (v/v) carbonated calcium hydroxyapatite crystals. This is in contrast to bone and dentine, which comprise 10% and 13% (v/v) carbonated calcium hydroxyapatite crystals, respectively. In addition, enamel crystals are larger, more uniform in morphology and more highly crystalline than their counterparts in bone or dentine. The disposition of enamel crystals within the tissue is highly ordered, with their long *c*-axes running approximately parallel and forming the 5–7 μm diameter enamel prisms, or rods, which are interspersed by interprismatic crystals. The mechanisms of crystal initiation and subsequent control of crystal growth and morphology are central problems in enamel formation.

Enamel formation proceeds via a series of distinct chemically and histologically defined stages (summarized in Fig. 1), i.e. secretion, transition and maturation (Robinson et al 1981a). During the secretory stage, the enamel-forming cells (the ameloblasts) exhibit a tall columnar morphology and secrete an extracellular protein-rich matrix as they migrate away from the dentine. Enamel crystals precipitated in the correct architectural arrangement then grow in length (*c*-axis growth) in the wake of the retreating ameloblasts. During the transition stage, the ameloblasts begin to reduce

in height and lose their secretory characteristics; matrix secretion ceases, and protein is withdrawn and replaced by tissue fluid. At the onset of the maturation stage, the ameloblasts are 50% of their original height and they begin to demonstrate characteristic membrane specializations. During this stage the enamel crystals grow significantly in both width and thickness as the bulk of the mineral present in the mature tissue is deposited.

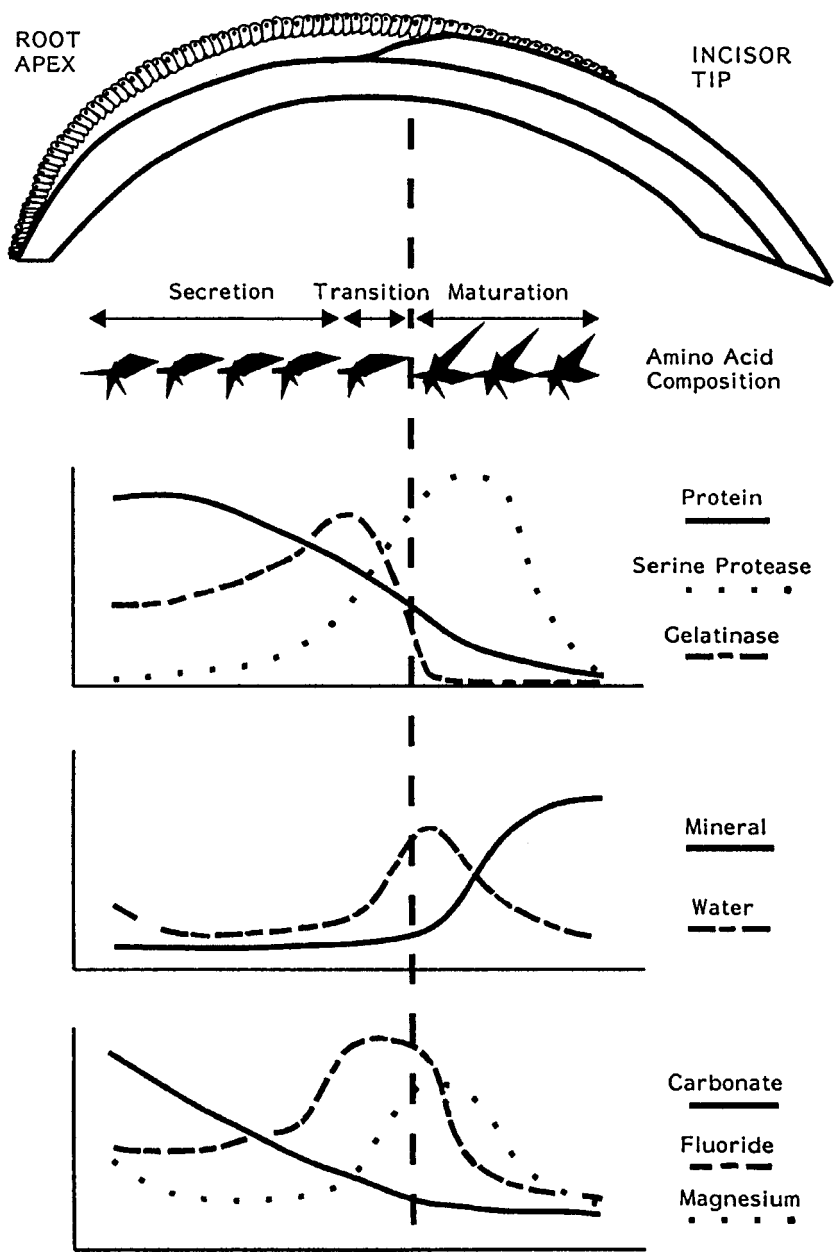
Although the bulk of the mineral present in mature enamel is deposited as the enamel crystals grow in width and thickness during the maturation stage as described above, limited growth in these dimensions also occurs during the transition and secretory stages (Daculsi & Kerebel 1978). This is particularly evident in the older, deeper layers of thicker enamels (e.g. human, cow and pig enamels, which can be more than 1 mm in thickness). In this respect, these deeper enamel layers, although present in enamel defined as secretory or transition stage, might be more akin to maturation stage enamel. 'Maturation' processes described in this presentation will therefore include changes that precede the classically defined maturation stage itself.

Nature of the extracellular mineral phase

Figure 1 also provides an overview of the changes in the chemistry of the extracellular mineral phase that take place during enamel development. The mineral phase of enamel comprises carbonated hydroxyapatite crystals that extend the full width of the tissue from the amelodentinal junction to the surface limit of the tissue. During the secretory stage, the mineral contains relatively high concentrations of carbonate. However, carbonate concentration falls towards the maturation stage, presumably as the surface area:volume ratio of the crystals decreases and mineral with a lower carbonate content is acquired (Hiller et al 1975). In contrast, both fluoride (Weatherell et al 1975) and magnesium (Robinson et al 1984) concentrations increase during the transition/early maturation stage. This may be due to greater permeability of the enamel organ at this stage or a consequence of altered cellular activity. The relevance of these changes in mineral chemistry is discussed later.

Nature of the extracellular protein matrix

The extracellular matrix of developing enamel is presumed to control crystal growth and morphology, and to provide an instructional template that dictates the gross architectural arrangement of the crystals in the tissue. During the secretory stage (where crystal elongation predominates over growth in width and thickness), ordered extracellular processing of nascent protein generates a complex milieu of matrix components (reviewed by Brookes et al 1995). However, this protein matrix is ephemeral, being degraded and completely removed during the transitional and maturation stages (Robinson et al 1975). As the protein matrix is lost, it is replaced with tissue fluid in which the enamel crystals grow in width and thickness until most of the tissue volume is occluded with mineral.



The extracellular protein matrix comprises several distinct protein species, which are outlined below.

Amelogenins. These are proline-rich proteins that dominate the secretory stage enamel matrix. The amelogenin population is heterogeneous (Fincham et al 1979), despite being derived from a single gene (with copies on the X and Y chromosomes in some species), due to alternative splicing of amelogenin mRNA and extracellular processing of the nascent proteins (Gibson et al 1992, Simmer et al 1994).

Proline-rich non-amelogenins. These comprise approximately 10% of the total protein matrix and can be divided into two immunologically distinct groups: 13–17 kDa non-amelogenins (amelins, ameloblastins or prism sheath proteins) (Uchida et al 1995); and 32–142 kDa non-amelogenins. These were recently shown to be serine proteases capable of degrading amelogenins (Uchida et al 1991, Moradian-Oldak et al 1996).

Other enzymes. A range of other proteases responsible for matrix processing and/or degradation include metalloproteases and serine proteases (Overall & Limeback 1988, DenBesten & Heffernan 1989, Robinson et al 1990, Tanabe et al 1992), in addition to alkaline phosphatase (Moe et al 1986, Robinson et al 1990) and carbonic anhydrase (Kakhei & Nakahara 1985).

Extraneous serum proteins. A range of serum proteins have been detected in developing enamel (Limeback et al 1989, Strawich & Glimcher 1990), the most notable of which is serum albumin (see below).

The processes surrounding crystal growth culminating in the massive mineral uptake during classical maturation have been the main theme of our investigations. Our strategy has been to use micro-sampling and micro-chemical analysis to determine

FIG. 1. (*opposite*) Summary of the stages of enamel development in the continuously erupting rat incisor. Secretory-stage ameloblasts are tall, polar cells with a characteristic Tomes' process at their distal end. During transition, the ameloblasts begin to shorten and the Tomes' process is lost. Maturation-stage ameloblasts may be reduced in height by 50% and show modulation between ruffled and smooth distal borders. The amino acid composition of the enamel matrix protein (shown as rose diagrams [Robinson et al 1975]) shows a marked change at the beginning of the maturation stage with an obvious loss of proline, glutamine and leucine, which are all characteristic of amelogenin. This selective loss is concomitant with a massive decrease in protein content within the tissue. Gelatinase activity, indicative of neutral metalloproteases, is obvious throughout secretion but is greatly reduced in transition and maturation. In contrast, serine proteases with amelogeninase activity show an apparent up-regulation at the beginning of the maturation stage. The mineral content per volume of the enamel remains relatively constant during the secretory and transition stages but increases dramatically during maturation, reflecting crystal growth in width and thickness. Water and extraneous ions such as magnesium and fluoride tend to accumulate at the transition/maturation border, whereas mineral carbonate concentrations decrease steadily throughout development. Relative values are shown for each component.

the chemical changes occurring throughout each developmental stage of the continuously growing rat incisor.

The role of inorganic ions during maturation

The growth of apatite crystals during enamel development is accompanied not only by changes in size and morphology but also by changes in crystal chemistry. For example, carbonate concentration decreases as enamel development proceeds (Hiller et al 1975), presumably reflecting the metabolic activity of the ameloblasts. Carbonate is known to retard crystal growth and to produce a less well-ordered apatite. In addition, recent work in our laboratories has demonstrated that carbonate is readily taken up by developing enamel crystals *in vitro* and promotes specific growth in crystal width compared with thickness (see Fig. 2). This raises the possibility of a level of control that is independent of the organic matrix.

Magnesium ions increase during the classical maturation stage (Robinson et al 1984) and this is probably due to the relatively free access of diffusible ions into the enamel

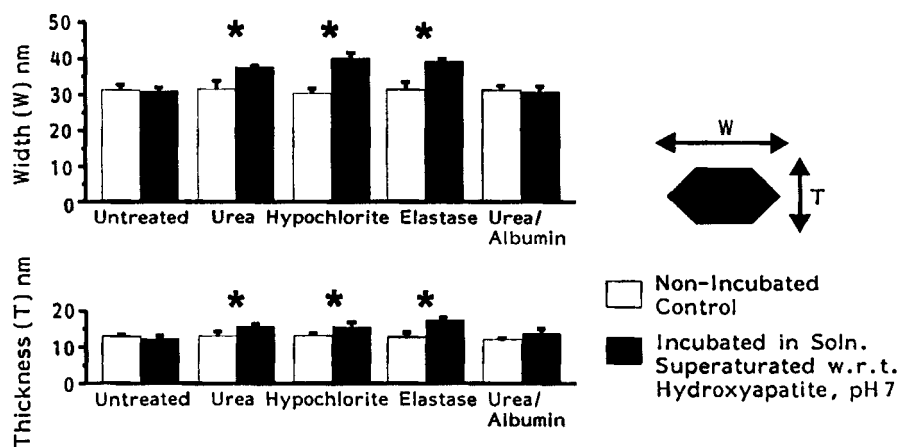


FIG. 2. The effect of proteins on the control of enamel crystal growth *in vitro*. We measured early maturation-stage crystals from developing rat enamel directly using transmission electron microscopy coupled with image analysis before and after incubation for seven days in solutions supersaturated with respect to hydroxyapatite at pH 7.0. No significant growth was observed in either width or thickness of the crystals. Removal of residual matrix proteins using 8 M urea before incubation resulted in a significant growth in both dimensions, indicating that endogenous enamel proteins can inhibit crystal growth. Treatment with hypochlorite to remove matrix proteins resulted in a fivefold increase in the mineral carbonate content; significant growth was observed after incubation, with preferential growth in width. Treatment with a serine protease (elastase) prior to incubation also produced significant crystal growth in both width and thickness. However, if we incubated crystals in a 4% albumin solution after removal of endogenous protein using 8M urea, no growth was observed after incubation, suggesting that serum albumin is capable of inhibition of enamel crystal growth. * $P < 0.05$.

during transition. Magnesium is also known to slow down crystal growth and to produce less well-formed crystals (reviewed by LeGeros 1991), and it is therefore again possible that a modulating effect occurs during the maturation stage.

Role of the organic matrix (amelogenin) during maturation

Amelogenin protein biochemistry

Many complex changes occur within the extracellular protein matrix during enamel development and a review of these biochemical alterations is a necessary prerequisite for the understanding of enamel crystal growth. This has recently been carried out by Brookes et al (1995) with respect to the major protein species, the amelogenins (see Fig. 3). Parent amelogenin molecules are secreted into the newly formed outer surface layer of the developing tissue. Although there is accumulating evidence for a number of alternatively spliced products, the dominant parent amelogenin corresponds to a six exon amelogenin mRNA transcript. This molecule (generically referred to as the 25 kDa amelogenin) is hydrophobic except for a short sequence at the C-terminal. However, this hydrophilic C-terminal (teleopeptide) is enzymically cleaved soon after secretion. The resulting 23 kDa amelogenin is further processed to generate a 20 kDa amelogenin that appears to accumulate in the subsurface enamel and forms the major component of the extracellular protein matrix. Further processing of

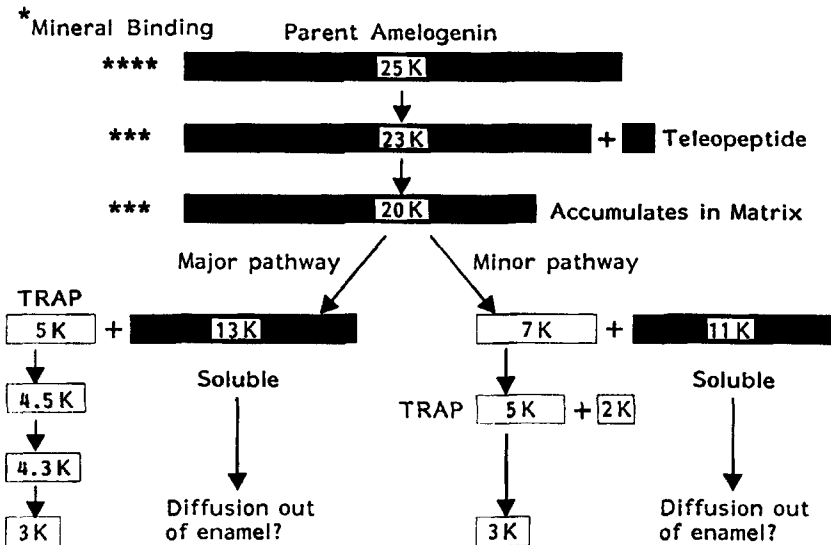


FIG. 3. Suggested sequence of amelogenin breakdown during porcine enamel development. Molecular weights shown are based on SDS-PAGE data (adapted from Brookes et al 1995). Components shown to bind to the mineral under *in vivo*-like conditions are indicated. TRAP, tyrosine-rich amelogenin protein.

the 20 kDa amelogenin occurs by removal of its tyrosine-rich N-terminal, generating a 5 kDa tyrosine-rich amelogenin protein (TRAP) and a peptide that is soluble in the secretory stage tissue fluid (enamel fluid).

Amelogenin-processing enzymes

The carefully orchestrated series of extracellular processing events described above indicate a role for proteolytic enzymes in the control of matrix degradation. Using a zymographic technique (Robinson et al 1990), we have investigated gelatinase and amelogeninase activity at each developmental stage in rat incisor enamel (Fig. 1). Gelatinolytic neutral metalloendoprotease activity seemed to occur principally during the secretory stage, appearing as a doublet migrating at approximately 65 kDa. These enzymes were incapable of degrading amelogenin substrate to peptides small enough to diffuse out of zymographic gels. Similar enzymes have been shown to cleave the hydrophilic teleopeptide from the parent amelogenin (Tanabe et al 1992, Moradian-Oldak et al 1994). This activity would generate a product large enough to be stained during zymography and would therefore not be detectable using the zymographic technique. Amelogenolytic serine protease activity became evident during the transition and maturation stages. This enzyme, which migrated at approximately 30 kDa produced cleared areas on amelogenin substrate gels, indicating that it was capable of degrading amelogenin to small soluble fragments. Other studies have indicated that a 30 kDa serine protease is present in secretory stage porcine enamel (Tanabe et al 1992). However, this activity was only detectable in the deeper layers of secretory stage enamel, reinforcing the suggestion that the deeper (and thus older) layers of secretory stage enamel in thicker tissue resemble the maturation stage proper.

It is generally agreed that the metalloendoproteases serve to process nascent amelogenins, giving rise to discrete amelogenin fragments that form the secretory stage enamel matrix. In contrast, the serine proteases appear to function to degrade these amelogenin fragments further to facilitate their complete removal during the maturation stage. This removal of the matrix *in vivo* coincides with the onset of the major period of mineral deposition (crystal growth in width and thickness) and the spatial and temporal regulation of the expression of these enzymes is therefore presumably central to normal enamel development.

Function of the organic matrix in crystal growth

Initiation of enamel crystal growth

Although not strictly a maturation process, a brief mention of the process by which enamel crystal growth is initiated is apposite. Structural studies which have physically linked the initiation of crystals with dentinal collagen or dentine crystals prompted the suggestion that nucleation/initiation of enamel crystal growth occurs at the dentine

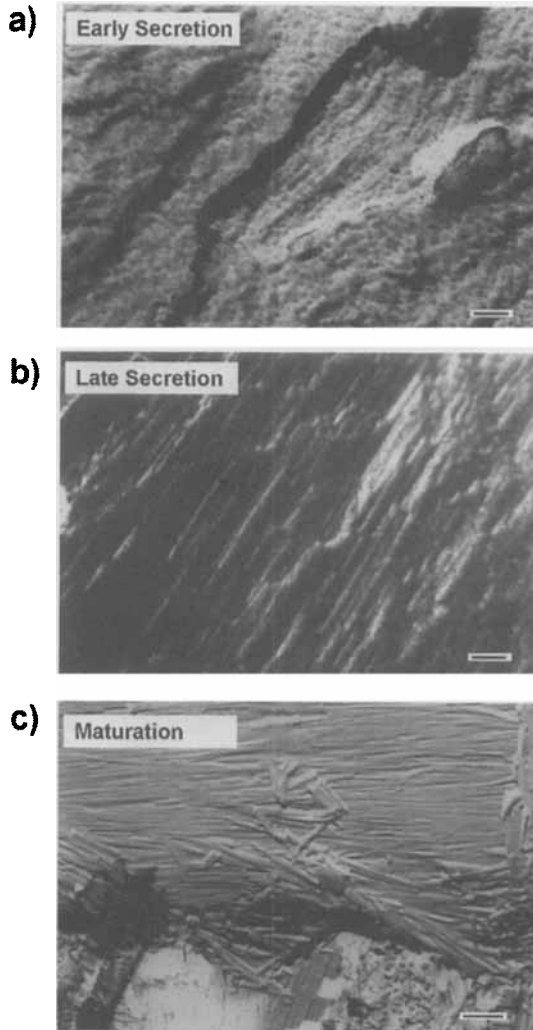


FIG. 4. Transmission electron micrographs of freeze-fractured developing rat incisor enamel at different stages of development. (a) Early secretory enamel. Bar = $0.25\ \mu\text{m}$. (b) Late secretory enamel. Bar = $0.25\ \mu\text{m}$. (c) Maturation stage enamel. Bar = $0.5\ \mu\text{m}$. Co-linear spheres of organic material can be seen in the secretory stage tissue, suggesting the presence of initiation/nucleation sites within the matrix. These are no longer apparent in the maturation stage, where controlled crystal growth can be seen to have occurred in the form of well-organized prisms containing large crystals.

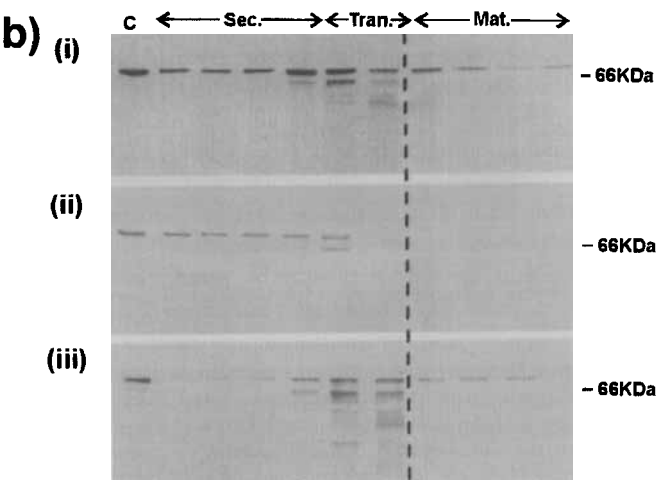
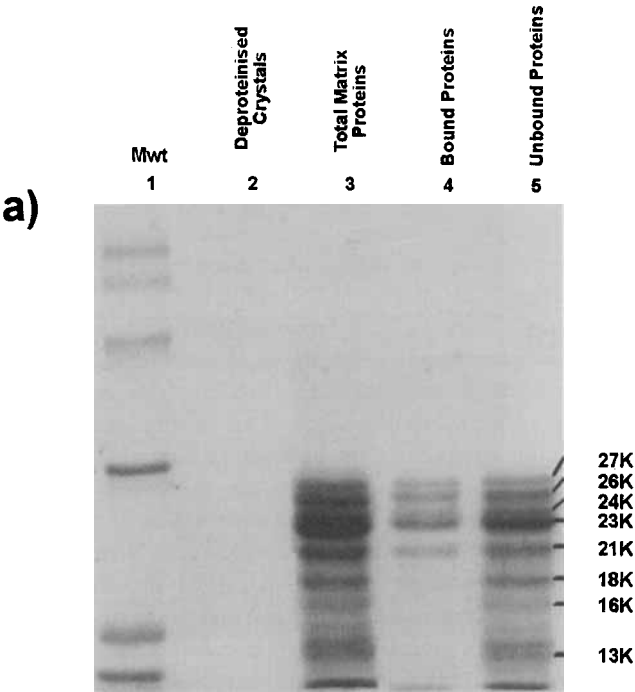


FIG. 5. (*opposite*) (a) SDS gel electrophoretograms showing selective binding of enamel matrix proteins extracted from secretory stage rat incisor enamel matrix to deproteinized secretory stage rat incisor enamel crystals. Lane 1: molecular weight markers. Lane 2: proteins extracted from deproteinized secretory stage enamel crystals. The deproteinization involved sequential extraction of secretory stage tissue with 0.1 M phosphate buffer (pH 7.4) followed by 50 mM Tris/4 M urea (pH 7.4). We demineralized these crystals using acetic acid and desalted the extracts prior to SDS-PAGE. No proteins were detected, suggesting that the secretory stage crystals were completely deproteinized. Lane 3: total matrix proteins extracted from secretory stage rat incisor enamel used in binding experiments. We demineralized secretory stage rat incisor enamel by dialysis against 10% acetic acid (3 kDa cut-off) and recovered the extracted proteins by freeze drying. Proteins in the range of <10–27 kDa could be detected. Lane 4: proteins associated with the crystals following incubation of deproteinized secretory stage enamel crystals with total matrix proteins. We incubated synthetic enamel fluid (pH 7.28) (Aoba & Moreno 1989) for 45 min at 0–4 °C. Following incubation, we centrifuged the crystal preparations and removed the supernatant. We washed the crystals several times and demineralized them using acetic acid to release mineral-bound proteins. Selective uptake of components in the higher molecular weight range can be seen. Lane 5: proteins remaining in the supernatant following incubation with deproteinized enamel crystals as described above. Note that the molecular size of amelogenin in the rat is slightly larger than that of porcine amelogenin, on which the molecular weights discussed in the text (and shown in the schematic diagram in Fig. 3) are based. The corresponding molecular weights in the porcine tissue are as follows: rat 27 kDa amelogenin = porcine 7 exon transcript; rat 26 kDa amelogenin = porcine 6 exon 25 kDa parent amelogenin; rat 24 kDa amelogenin = porcine 23 kDa amelogenin (produced by removal of teleopeptide from 25 kDa parent amelogenin); rat 23 kDa amelogenin = porcine 20 kDa amelogenin produced by further C-terminal processing of the teleopeptide-depleted 23 kDa amelogenin. The bulk of the remaining proteins present in the rat enamel matrix extract presumably correspond to amelogenins produced by further processing of the molecules described above in addition to alternatively spliced products.

(b) Western blots showing presence of albumin in rat incisor enamel at the secretory (Sec), transition (Tran) and maturation (Mat) stages of development. We separated proteins using SDS-PAGE, transferred them to nitrocellulose sheets and probed them using polyclonal antibodies raised against rat serum albumin. (i) Distribution of albumin at each developmental stage following extraction of proteins using 0.1 M phosphate buffer, pH 7.4, which is known to desorb mineral-bound albumin. Intact albumin (molecular mass = 66 kDa) was detected throughout the secretory stage with evidence of degradation at transition. Only very small amounts of albumin were detectable in the maturation stage. (ii) Distribution of albumin at each developmental stage following extraction of proteins using synthetic enamel fluid (Aoba & Moreno 1989). The results show that albumin is soluble in the enamel fluid during the secretory stage. Lack of cross-reactivity in the late transition/maturation stage indicates that albumin becomes mineral bound in the tissue at this point. (iii) Distribution of albumin at each developmental stage following extraction of proteins using 0.1 M phosphate buffer after prior extraction with synthetic enamel fluid as described above. The results clearly show that albumin in the transition/maturation stages is mineral bound. The association of albumin with the enamel crystals coincides with the removal of bulk matrix proteins *in vivo*, indicating a possible protective role for the developing enamel matrix. C, positive control (rat serum).

surface (Arsenault & Robinson 1989). Alternatively, freeze-fracture studies carried out at different stages of enamel development have suggested that a linear series of nuclei may fuse to form the developing enamel crystal (Fig. 4a) (Robinson et al 1981b).

Inhibition/modulation of crystal growth

Inhibition of crystal growth at specific crystal surfaces could modulate both crystal size and morphology. The effect of endogenous protein on enamel crystal growth has been tested *in vitro* by incubating early maturation stage rat incisor enamel crystals in solutions supersaturated with respect to hydroxyapatite at pH 7.0 (Robinson et al 1989). Direct measurement of crystal width and thickness indicated that little or no crystal growth occurred. However, when protein was removed with urea, hypochlorite or serine proteases (chymotrypsin and elastin) prior to incubation, significant growth in both width and thickness occurred (Fig. 2) (Robinson et al 1990). This indicated that endogenous matrix components could have an inhibitory effect on crystal growth. Their removal *in vivo* by the serine proteases active during the transition/maturation stage appears to be a necessary pre-requisite for growth to occur.

Efforts have been made to identify specific matrix proteins that are able to bind to developing enamel crystals and thereby potentially influence their growth. We have incubated deproteinized developing rat incisor enamel crystals with secretory stage matrix proteins in a solution corresponding to the reported composition and pH (7.28) of the enamel fluid *in vivo*.

Five protein components, in the range of 21–27 kDa, were selectively adsorbed onto the enamel crystals (Fig. 5a). All of these components had the amelogenin N-terminal amino acid sequence. Further identification was based on relative molecular size and abundance of a particular species in the tissue. From these results it would appear that under *in vivo*-like conditions, the parent 25 kDa amelogenin (26 kDa in the rat), the 23 kDa amelogenin (i.e. the parent molecule minus the hydrophilic teleopeptide; 24 kDa in the rat) and the 20 kDa processing product (23 kDa in the rat) are all capable of binding to deproteinized enamel crystals.

Previous work by Aoba et al (1987) using synthetic apatite crystals and porcine enamel matrix proteins dissolved in acetate buffer at pH 6.0 reported that the 25 kDa parent amelogenin was selectively taken up by the mineral in preference to amelogenin-processing products. However, when Aoba et al (1987) conducted the same experiment at pH 7.8, a similar binding pattern to that shown in Fig. 5a was observed.

It would appear, therefore, that the 25 kDa parent amelogenin and its immediate processing products — including the most dominant matrix component (the 20 kDa amelogenin) — could influence crystal growth by adsorption onto crystal surfaces. However, since the 25 kDa parent amelogenin is spatially restricted to the superficial layers of the enamel, it could presumably only influence crystal growth near the ameloblasts where crystal elongation is occurring. Amelogenin-processing products are therefore more likely to inhibit/control lateral crystal growth, particularly in the

deeper, older enamel where they are the dominant proteins present. The increased crystal width and thickness found in the deeper layers of secretory stage tissue of thicker enamels (e.g. porcine enamel) could be explained by the action of the 30 kDa serine proteases degrading inhibitory amelogenin processing products, thus allowing for crystal growth in the deeper enamel. However, the crystals in the deeper layer of the enamel are also 'older' and may have been growing for longer than the 'younger' crystals nearer the surface. These two possibilities are not mutually exclusive.

Support scaffolding

The developing enamel matrix surrounding the immature crystals may serve to direct crystal growth, determining the final tissue architecture. Unfortunately, there are limited data available describing the physical structure of the solid state developing enamel matrix and how this might direct crystal growth. However, ultrastructural observations have reported the presence of reticular organic structures (Travis & Glimcher 1964, Jessen 1968, Warshawsky 1971). In an effort to avoid the structural artefacts that often accompany demineralization, fixation and staining, Robinson et al (1981b) used a freeze-fracture technique to investigate the ultrastructure of developing rat incisor enamel. This revealed the loss of amorphous (presumably organic) material from the developing crystal surfaces during the transition/maturation stage, supporting the view that massive matrix loss begins at this stage (Fig. 4). This amorphous material was composed of 30–50 nm diameter spheres that were aligned along the long *c*-axes of the crystals and could represent aggregates of insoluble amelogenin molecules. It is therefore possible that these spheres could fulfil not only a packing role—occupying space within the tissue that would eventually be replaced first by fluid and then by growing apatite crystals—but could also direct *c*-axis growth during the secretory stage. More recent work using a recombinant 25 kDa parent amelogenin analogue *in vitro* has supported this view (Moradian-Oldak et al 1995). Small, mono-disperse 15–20 nm spheres have been observed in the absence of mineral. Such structures may only be present in the outer layers of the enamel *in vivo*, however, as extracellular processing of the 25 kDa parent amelogenin rapidly produces the 23 kDa and then the major 20 kDa components. When a recombinant 23 kDa amelogenin (i.e. minus the teleopeptide) was used in similar experiments, much larger aggregates were formed instead of the mono-dispersed spheres. It is not known whether the spherical structures formed by the 25 kDa parent amelogenin remain stable following enzymic removal of the teleopeptide *in vivo*.

Protection (prevention of the access of growth inhibitors to crystal surfaces)

Recent work in our laboratory has demonstrated that components which are not normally bound to the mineral *in vivo* can become bound when bulk matrix proteins are removed by denaturing solvents (see Fig. 5b). Using selective extraction procedures, albumin (a known inhibitor of enamel crystal growth [Fig. 2]) was

shown to be soluble in secretory stage enamel but mineral bound at the beginning of the maturation stage following removal of the amelogenin-rich matrix. This raises another important aspect of the role of endogenous extracellular matrices in mineralized tissues, which may be to protect the crystal surfaces from potential inhibitory molecules. *In vivo*, albumin is subsequently degraded and removed from the maturation stage tissue by the proteases that are active during this stage (see above). This activity may be regarded as an essential scavenging system which operates during the maturation stage to remove any proteins/peptides remaining in the tissue that could potentially inhibit final crystal growth.

Role of the organic matrix in enamel dysplasias

Many enamel dysplasias of genetic and non-genetic origin share a common phenotype, presenting as white porous tissue characteristic of incompletely mature tissue. One possible cause for this would be a failure in the mechanisms responsible for the removal of inhibitory proteins during development. This could be related to ineffective enzyme activity and/or to increased affinity/binding of inhibitory protein to the mineral phase. Inhibition of crystal growth has been implicated as a causative factor in both fluorosis and hypomaturation amelogenesis imperfecta (Wright 1995). However, many apparently diverse conditions can give rise to enamel dysplasia, including mechanical trauma and diseases of childhood. One common feature of all of these conditions is the likelihood of transient hyperaemia during enamel development, leading to leakage of albumin into the tissue (Robinson et al 1992). If this occurs during the late maturation stage, or in such quantities as to overwhelm the enzymes responsible for matrix removal, impaired crystal growth would be expected, resulting in the eruption of incompletely mature tissue. In support of this, both mechanical damage and hypobaric hypoxia have been shown to produce incompletely mature enamel that contains substantial amounts of albumin in the rat model. Albumin has also been demonstrated in the dysplastic enamel from patients suffering from the junctional form of epidermolysis bullosa (Strafford et al 1994).

Acknowledgements

The authors thank the Medical Research Council and the Wellcome Trust for their continued support of this work and C. L. Godfrey for invaluable assistance with the illustrations.

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DISCUSSION

Slavkin: Over 30 years ago, in collaboration with Richard Greulich, we used the then new tool of autoradiography applied to tooth development and enamel formation (Greulich & Slavkin 1965). We injected a number of different radiolabelled amino acids into young rodents *in vivo*, and observed a pattern of diffusion for each labelled precursor through the enamel, from the cell surface to the dentine–enamel junction (DEJ). In contrast, in dentine and bone formation there were always discrete lines of radioactivity like rings of growth in a tree. Therefore, it was thought that there was an incremental pattern in dentine and bone formation, whereas enamel formation was a thixotropic gel-like matrix. The model that you presented suggests precursor molecule-selective processing and enzyme-mediated control. How does this fit with the alternative splicing of different precursors at different stages in tooth development, and with this older set of observations of rapid diffusion of labelled proteins through the forming enamel matrix?

Robinson: It fits perfectly well because smaller soluble molecules will diffuse through the enamel as the solid phase starts to disintegrate and the matrix becomes more rarefied. As the larger nascent molecules degrade into soluble molecules they will begin to diffuse through the more rarefied matrix and carry the radiolabel with them. As for the alternative splicing, until we know how many of the molecules there are and have more of an idea about their processing pattern it is not possible to answer that question. The processing, however, is likely to follow a similar pathway provided the same enzyme cleavage sites are present.

Snead: You seemed to exclude alternative splicing in considering the stoichiometry of the matrix but insisted on assuming that the dominant protein was a 20 kDa

molecule. You said that the amount of material would then be constant. However, the gel you showed suggests that proteins of a higher molecular mass are not equally represented and that most of the proteins have a small molecular mass.

Robinson: Yes, there is a shift to smaller sizes during secretion. However, a distinct breakdown pattern is observed in that the 27 kDa protein is processed rapidly to the 20 kDa protein, which apparently breaks down less quickly because it then accumulates.

Simmer: Couwenhoven & Snead (1994) and Gibson's group (Yuan et al 1996) have shown that albumin is not expressed by ameloblasts. Earlier studies in the rabbit showed that perfused radiolabelled albumin does not enter the enamel (Kinoshita 1979). Given the doubt raised by these results as to the presence of albumin in the developing enamel matrix, it seems to me that techniques less likely to induce post-mortem bleeding, such as immunohistochemistry, are required to ensure that the observed serum albumin is not an artefact.

Robinson: The observation that albumin is not expressed locally doesn't matter. For example, hormones are made at a location distant from the tissue that they affect. We always find albumin in freeze-dried enamel, particularly during maturation and less so during secretion, despite the fact that we have gone to great lengths to exclude any kind of contamination; for example, by freeze drying the tooth before extraction.

Simmer: Immunohistochemistry may be preferable.

Robinson: There's no reason to suppose that the degree of contamination would be any less because it would have to be fixed, and molecules do move during the fixation process.

Rosenbloom: We've spent a long time studying bovine alternative splicing. The relative amounts of alternatively spliced mRNAs are much less than the full-length amelogenin transcript. Do you also observe this in rodents?

Simmer: The relative abundance of the smaller amelogenin isoforms varies depending upon the technique used. We generally use reverse transcriptase PCR techniques. LRAP (the 59 residue leucine-rich amelogenin protein) is fairly abundant. It is about 10–20% of the level of the principal (180 residue) amelogenin. Using immunochemistry, we have found that rat amelogenins that contain the amelogenin exon 4-encoded segment are just 1–3% of the whole.

Fincham: We have been unable to find LRAP in mice, which may purely be for technical reasons. However, the principal amelogenein, i.e. the full-length amelogenin that does not contain exon 4, is undoubtedly dominant. In the case of pig enamel, it is extremely easy to isolate the abundant 25 kDa form and the 23 kDa and 20 kDa forms using a high performance liquid chromatography column. It is not so easy to find the other fragments, but we have detected them using mass spectrometry. Therefore, in all the species we've looked at—human, cow, pig, rat and mouse—the single large amelogenin is dominant, and the processed products are much more difficult to find.

Winter: You mentioned hypoxia. Nikiforuk & Fraser (1981) suggested that this effect is only produced by interfering with calcium homeostasis, such as in hypocalcaemia. Thirty-seven years ago similar effects were reported in patients with

hyperbilirubinaemia (Miller & Forrester 1959). Have you looked at the effects of acidosis on the level of serum calcium?

Robinson: No, we have not yet looked at calcium availability. Albumin is, however, present in many of these conditions and it definitely inhibits crystal growth. I'm not saying that this is the only answer but it is there and it can inhibit crystal growth. A defect in calcium transport could not of course be excluded.

Slavkin: Many enzymes are synthesized and secreted before they become active, and environmental factors subsequently activate them. Are you suggesting that serum albumin is a systemic protease inhibitor, influenced by temperature, hypoxia or systemic diseases, which can modulate proteolytic activity in a tooth? And could albumin be a competitive substrate in other enzyme systems?

Robinson: Albumin could be a competitive substrate for local enzymes. However, all I'm saying is that a disturbance at the maturation/transition stage leading to albumin uptake results in immature enamel.

Slavkin: Are you suggesting that inactive enzyme molecules can't diffuse from the blood supply through the papillary layer to the matrix, where they act as protease inhibitors?

Robinson: No, that is only one facet of the situation. The process occurs over a long period of time and, at some point, the enzymes may not, for whatever reason, be able to cope with the albumin that is present. We have proposed that the final destruction of amelogenin and any other protein present by the serine proteases is a mechanism to cope with this. Whether albumin is present or not naturally, i.e. if it is introduced by trauma, for example, it's likely to have the effect of impairing maturation.

Slavkin: This effect could be produced by any number of molecules.

Boyde: Was all the material you looked at deciduous?

Robinson: Yes.

Boyde: The surface enamel of most rodent incisors contains iron pigment, which is deposited after the end of maturation. You have disrupted maturation by introducing albumin into the enamel, but it is unclear as to whether you have also disrupted the final differentiation stage of the ameloblasts so that they do not transport iron into the outer enamel layer. The teeth looked white, so presumably iron was not present.

Robinson: We did not examine this.

Mann: Do the crystals initially grow in a lateral dimension, close to the DEJ, and then thicken? This would suggest that diffusion processes are operating in the 100 μm mineralization zone.

Robinson: In the pig, at least, there is obvious crystal growth near the dentine at the secretion stage. However, this is not so obvious in other species. The thickness of the enamel and the time taken to secrete it have to be taken into account. I have tried to give a reference mark, but it's a blurred one because the maturation process does not occur at exactly the same time in all the cells.

Mann: Presumably, a condition could develop in which the layers closest to the retracting cell become heavily mineralized compared with other regions where no mineralization occurs, i.e. a progressive mineralization back to the DEJ.

Robinson: This is possible and it is probably why most of the matrix is removed progressively starting near the dentine.

Mann: But diffusion would still occur.

Robinson: This could seal the surface.

Mann: Exactly, and it doesn't do that, so that's my point.

Fejerskov: Concerning the white/opaque boundary and its relative position with respect to the cell differentiation, you quite rightly mentioned that, at least in humans, it is a continuum from late enamel secretion to maturation. Where is the position of the enamel organ relative to the opaque boundary, when the Tomes' processes are lost? In other words, is what you call transition synonymous with the definition of transition of a stage at which cells 500 μm in length are observed?

Robinson: Yes I think so. We freeze dried bovine and rodent teeth, then took the cells off and looked at the enamel underneath. At the white/opaque boundary the cells have finally shortened and have lost their Tomes' processes. Immediately preceding this point is the transition stage, which is about 1–2 mm, depending on the species, and corresponds to a stage where ameloblasts are shortening and Tomes' processes are disappearing (Robinson et al 1981).

Nanci: This fits with our results, which suggest that the white/opaque boundary lies within the maturation zone (Smith & Nanci 1989).

Fejerskov: But your calculations were about 2000 μm into the zone of maturation, which makes sense because Weile et al (1993) demonstrated that the white/opaque boundary is definitely within the cellular domain of maturation ameloblasts.

Simmer: Takashi Uchida has observed that, during maturation, the protein closest to the ameloblasts disappears first and the protein closest to the DEJ disappears later (Uchida et al 1995). This raises the question as to how enamel proteins are removed. How important is the removal of enamel proteins through the intercellular space as the intercellular junctions form and disassemble during ameloblast modulations?

Nanci: We have observed exactly the opposite; that is, maturation and bulk protein degradation starts at the DEJ (Nanci et al 1992). In addition, using immunocytochemistry and radioautography, we have not obtained any conclusive evidence showing a major uptake of intact or nearly intact enamel proteins by ameloblasts during the maturation stage. We believe, at this point, that enzymes clip the proteins into small fragments which diffuse out of the enamel layer. Some of these fragments may be picked up by ameloblasts along their basolateral surfaces but we have no evidence for the bulk uptake enamel proteins at their apical surface (reviewed in Nanci & Smith 1992).

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Inherited enamel defects

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Abstract. This paper describes the clinical, histological and genetic findings in individuals with amelogenesis imperfecta diagnosed in more than 50 families in the county of Västerbotten, northern Sweden. Using pedigree analysis, families with autosomal and X-linked inheritance as well as sporadic cases of amelogenesis imperfecta have been recognized. A clinical subclassification in eight different variants of amelogenesis imperfecta has been made. The gene defects have been identified for two of these variants and the chromosomal location has been established for a third variant.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 175-186

Inherited enamel defects, i.e. amelogenesis imperfecta, are characterized by clinical and genetic heterogeneity. Since tooth enamel is of ectodermal origin, amelogenesis imperfecta can be a symptom in syndromes involving other ectodermal tissues such as hair, skin, nails and parts of the eye (Pindborg 1982). It is, however, generally accepted that the term amelogenesis imperfecta should be limited to those congenital defects affecting only enamel formation and not accompanied by morphological or metabolic defects in other body systems. In diagnosing amelogenesis imperfecta, relevant anamnestic data must therefore be obtained so that additional symptoms are revealed. Different prevalences of amelogenesis imperfecta have been reported, from 0.06 per 1000 in the USA (Witkop & Sauk 1976) to 1.4 per 1000 in northern Sweden (Bäckman & Holm 1986). The differences are most probably due to demographic factors and/or applied diagnostic criteria.

X-Linked amelogenesis imperfecta is caused by mutations in a gene encoding the enamel protein amelogenin, and it is likely that other forms of amelogenesis imperfecta are caused by mutations in additional genes involved in amelogenesis. The appropriate method of diagnosis and classification of amelogenesis imperfecta should be through the identification of these mutations (Aldred & Crawford 1995). At present this is possible only to a limited extent, and it is important to realize that the ideal method of classifying amelogenesis imperfecta is not yet established. Today, classification is based on clinical findings and on analysis of pedigrees. Thus, according to the most widely accepted classification scheme, four main forms of amelogenesis imperfecta can be distinguished, three of which are related to a certain stage in amelogenesis and the fourth connected with taurodontism. The four major forms can be further divided into

TABLE 1 Classification of amelogenesis imperfecta according to Witkop (1989)

<i>Type</i>	<i>Features</i>
I	hypoplastic
IA	hypoplastic, pitted autosomal dominant
IB	hypoplastic, local autosomal dominant
IC	hypoplastic, local autosomal recessive
ID	hypoplastic, smooth autosomal dominant
IE	hypoplastic, smooth X-linked dominant
IF	hypoplastic, rough autosomal dominant
IG	enamel agenesis, autosomal recessive
II	hypomaturation
IIA	hypomaturation, pigmented autosomal recessive
IIB	hypomaturation, X-linked recessive
IID	snow-capped teeth, autosomal dominant?
III	hypocalcified
IIIA	autosomal dominant
IIIB	autosomal recessive
IV	hypomaturation — hypoplastic with taurodontism
IVA	hypomaturation — hypoplastic with taurodontism, autosomal dominant
IVB	hypoplastic — hypomaturation with taurodontism, autosomal dominant

14 subtypes based on predominant clinical manifestation and mode of inheritance (Witkop 1989). Table 1 shows the classification of amelogenesis imperfecta according to Witkop (1989) which will be used in this paper.

A total of 51 families with amelogenesis imperfecta have been identified following a thorough search throughout the county of Västerbotten in northern Sweden (Bäckman 1988). The clinical and histological manifestations, mode of inheritance and the gene defects established to date in these families constitute the basis for this presentation.

Inheritance patterns and classification

Thirty-two of the 51 families studied demonstrate autosomal dominant inheritance of amelogenesis imperfecta. Six families demonstrate autosomal recessive and three families have X-linked inheritance. There are also 10 sporadic cases of amelogenesis imperfecta. Thus, autosomal dominant inheritance is the most prevalent, which is in accordance with results of previous studies (Witkop & Sauk 1976, Sundell & Valentin 1986). In the families with an autosomal recessive inheritance pattern, an alternative

possibility is autosomal dominant inheritance with reduced penetrance of the amelogenesis imperfecta gene in the parental generation. This could likewise explain amelogenesis imperfecta in the 10 sporadic cases or, alternatively, they could be new mutations. Taking these assumptions for granted and based on pedigree analysis, amelogenesis imperfecta is autosomally dominant or X-linked.

A clinical classification demonstrated the presence of eight subtypes of amelogenesis imperfecta. There are overlapping symptoms in most cases of amelogenesis imperfecta, i.e. the enamel can be both hypoplastic and hypomineralized but have a predominance of either symptom clinically (Wright et al 1992, Bäckman et al 1993). Therefore, subclassification was performed according to the predominant clinical manifestation. As a rule, both primary and permanent teeth are affected, but primary teeth are affected to a less severe extent than permanent teeth. Contrary to Witkop & Sauk (1976), but in accordance with later studies (Chosack et al 1979, Sundell & Valentin 1986), the hypoplastic variants are the most prevalent.

Hypoplastic amelogenesis imperfecta

Pitted hypoplastic amelogenesis imperfecta

Pitted hypoplastic amelogenesis imperfecta is the prevailing clinical manifestation in the 51 families with amelogenesis imperfecta, and these are connected with autosomal dominant inheritance in all but two families (Bäckman 1988). The pinpoint- to pinhead-sized pits are generally randomly distributed in the dentition, rather than chronologically distributed, and can therefore not be attributed to a certain stage in tooth development. Radiographically, the enamel contrasts normally to the dentine and is of normal extent. However, quantitative microradiography, i.e. transverse microradiography (TMR) imaging, has shown the mineral content to be somewhat lower than in corresponding non-affected teeth (Bäckman & Angmar-Månsson 1994). Qualitative microradiography shows hypomineralized areas at the bottom of the hypoplastic defects, and scanning electron microscopy (SEM) shows organic debris in the bottom of the pits, which could explain the extrinsic black staining almost always seen in adults with this clinical variant (Bäckman & Anneroth 1989, Bäckman et al 1989). One locus for autosomal dominant amelogenesis imperfecta has been mapped to the long arm of chromosome 4 by linkage analysis (Forsman et al 1994). However, in four families with pitted hypoplastic amelogenesis imperfecta, linkage to this region has been excluded (Kärman et al 1996a).

Local hypoplastic amelogenesis imperfecta

In local hypoplastic amelogenesis imperfecta, pits, grooves or one large hypoplastic area primarily in the middle third of the buccal enamel surfaces are observed in various positions and with a varying degree of severity both between affected individuals and between families. The tooth colour is extremely white but, with age,

yellow-brown discolourations are observed in connection with the hypoplastic defects. Areas of hypomineralization have been found connected with the hypoplasias (Sauk et al 1972a). In primary teeth, the extent of enamel is thinner than in corresponding non-affected teeth, but the mineral content is normal (Bäckman & Anneroth 1989, Bäckman & Angmar-Månsson 1994). The amelogenesis imperfecta phenotype is consistent across all affected individuals in the families examined and the inheritance pattern is autosomal dominant. Linkage analysis has located the gene for local hypoplastic amelogenesis imperfecta to the long arm of chromosome 4 (Forsman et al 1994, Kärman et al 1996a,b). The gene defect is so far not known, but the establishment of a yeast artificial chromosome over the region has prepared the way for the positional cloning of the gene (Kärman et al 1996b).

Rough hypoplastic amelogenesis imperfecta

Teeth with rough hypoplastic amelogenesis imperfecta have an extremely thin enamel layer so that teeth do not meet at a contact point. The tooth colour is yellow-brown and the mineral content is lower than in non-affected teeth (Bäckman & Angmar-Månsson 1994). SEM of a tooth surface shows crumbling of the brittle enamel and enamel prisms that are thereby exposed. Families with both autosomal dominant and autosomal recessive inheritance, as well as sporadic cases of amelogenesis imperfecta, have been examined. Linkage to the long arm of chromosome 4 could not be shown in families with this manifestation (Kärman et al 1996a).

A connection has been reported between a skeletal open bite relation and amelogenesis imperfecta (Rowley et al 1982, Persson & Sundell 1982, Bäckman & Adolfsson 1994). It has not been possible to explain the cause of the simultaneous occurrence of these two rare conditions because they involve tissues that are of a different developmental origin, i.e. ectodermal and ectomesenchymal. The problem remains unsolved in spite of the fact that growth and development are controlled by interactions between the two tissues. Comparisons of cephalometric data from cases with amelogenesis imperfecta and non-affected controls have shown statistically significant differences, indicative of a skeletal open bite relation in cases with rough hypoplastic amelogenesis imperfecta (Bäckman & Adolfsson 1994). This was also found in cases that did not have a dentoalveolar open bite relation.

Hypomaturation amelogenesis imperfecta

Snow-capped teeth

Snow-capped teeth represent a manifestation of amelogenesis imperfecta characterized by a lower degree of mineralization. The teeth look as though they have been dipped in white colour with an anterior to posterior distribution. Primarily the incisal/occlusal one-third to a half of the tooth is affected (Bäckman 1988). The hypomineralized areas

are not attributable to environmental factors, i.e. trauma, nor to a certain stage in tooth development. They look similar to fluorosis but they do not show accentuated perichymata and cannot be related to a high intake of fluorides. Radiographically, the extent of enamel is normal. By SEM it has been shown that only the surface of the enamel is affected. After etching of the surface with hydrochloric acid, a normal prism pattern is found (Escobar et al 1981). One hypothesis is that the most external prismless layer of the enamel, the end product of the ameloblast, is defective in this variant. As in previous studies (Winter & Brook 1975, Witkop & Sauk 1976), autosomal dominant and autosomal recessive inheritance patterns occur (Bäckman 1988).

Hypomaturation amelogenesis imperfecta

In teeth with hypomaturation amelogenesis imperfecta, the enamel is mottled opaque white with a yellow–brown discolouration that becomes more severe with advancing age. The enamel is of normal extent and radiographically of almost the same density as dentine (Bäckman 1988). TMR imaging shows a low mineral content primarily in the bulk of the enamel that is prone to detachment from the dentine, especially in the incisal/occlusal areas due to chewing forces (Bäckman & Angmar-Månsson 1994). That the incisal edge is actually broken away has been shown by SEM (Bäckman et al 1989). A disturbance causing reduced or cessation of protein removal during the maturation stage of development has been suggested to cause the elevated protein content of the enamel in these cases (Wright et al 1992). Linkage to the long arm of chromosome 4 can be excluded for one family with this manifestation (Kärrman et al 1996a).

Hypocalcified amelogenesis imperfecta

The enamel in teeth with hypocalcification amelogenesis imperfecta is characterized by a more severe degree of hypomineralization than that of teeth with hypomaturation amelogenesis imperfecta. The enamel is lustreless, opaque white to yellow–brown, of normal extent but so soft that it is often lost soon after eruption, leaving a tooth crown composed only of dentine. The cervical areas of the teeth seem better mineralized. Radiographically, the enamel fails to contrast with the dentine. These findings have been confirmed by TMR imaging (Bäckman & Angmar-Månsson 1994). Cephalometrically, this variant of amelogenesis imperfecta has been found connected with a skeletal open bite relation (Bäckman & Adolfsson 1994). The data for linkage analysis performed in two families with this manifestation are not conclusive (Kärrman et al 1996a). The LOD scores are weakly positive but too low to demonstrate linkage to the long arm of chromosome 4.

X-Linked amelogenesis imperfecta

Three families with an X-linked inheritance pattern have been examined. The clinical manifestation in one family is in accordance with X-linked hypoplastic amelogenesis

imperfecta, and X-linked hypomaturation amelogenesis imperfecta is manifest in the other two. In all three families different clinical manifestations are seen in affected women compared to affected men in the same family; a difference most probably attributable to the random inactivation of one of the X-chromosomes early in embryonic life (Lyon 1961). In X-linked amelogenesis imperfecta the enamel in men is generally affected, whereas the enamel in women also shows areas of normal enamel, thus reflecting the presence of normal and mutated ameloblasts.

Both X-linked variants were cephalometrically found to be connected to a skeletal open bite relation, with the possible exception of women with X-linked hypoplastic amelogenesis imperfecta. In this case, the individuals examined were too few for definite conclusions to be drawn (Bäckman & Adolfsson 1994).

The enamel in men with X-linked hypomaturation amelogenesis imperfecta is of normal thickness with approximately the same radiodensity as dentine. The colour is opaque white to mottled yellow-brown. In women with this manifestation, randomly alternating vertical ridges of normal translucent and opaque white to mottled yellow-brown enamel are seen. SEM examination of permanent teeth from both an affected woman and an affected man confirm this difference (Sauk et al 1972b). By linkage analysis, the gene for X-linked amelogenesis imperfecta has been mapped to region p22 of the X chromosome (Lagerström et al 1990). The region contains the locus for the amelogenin gene (Lau et al 1989). The amelogenin gene also has a functional Y homologue, although this is expressed at a reduced level (Salido et al 1992). The amelogenins, the principal secretory product of the ameloblasts, are involved in enamel matrix formation. In families with X-linked hypomaturation amelogenesis imperfecta, a large (5 kb) deletion has been found, with the consequence that almost the entire coding potential of the amelogenin gene is removed (Lagerström et al 1991). Qualitative and quantitative microradiography of primary molars from a boy with this manifestation show demineralized channels in the enamel, severe hypomineralization of the bulk of the enamel and a mineral content that is generally lower than normal (Bäckman & Anneroth 1989, Bäckman & Angmar-Månsson 1994). SEM shows voids in the enamel, indicating that enamel prisms are simply missing (Bäckman et al 1989). In spite of the large deletion in the amelogenin gene, some enamel has actually formed in men with X-linked hypomaturation amelogenesis imperfecta. One explanation is that amelogenin encoded by the gene on the Y chromosome is involved in enamel formation in these men.

Men with X-linked hypoplastic amelogenesis imperfecta have enamel that is extremely thin, brown to yellow-brown, smooth and shiny. Teeth do not meet at a contact point. The mineral content of the enamel seems to be in accordance with unaffected teeth (Wright et al 1992), and radiographically a thin enamel layer is seen outlining the teeth. In women with X-linked hypoplastic amelogenesis imperfecta, the severity of the manifestation varies, i.e. there are variations in the amount of enamel formed and the extension of the hypoplastic defects that show a vertical distribution. The gene defect in these families has also been mapped to region p22 of the X chromosome (Lagerström et al 1990). A deletion (9 bp) has been found in the signal

peptide of the amelogenin protein (Lagerström-Fermér et al 1995). The deletion most probably results in an impaired translocation of the amelogenin protein and could explain the clinical manifestation in this variant of amelogenesis imperfecta. Another four mutations in other parts of the amelogenin protein have been found (Aldred et al 1992a, Lench et al 1994, Lench & Winter 1995). Linkage to a second locus on the long arm of the X chromosome has been reported in a family with a marked variation in appearance both among affected men and women (Aldred et al 1992b).

Conclusion

It is obvious that mutations in different genes are involved in the causation of the different forms of amelogenesis imperfecta. Further, different mutations in the same gene are likely to produce different clinical manifestations as observed in X-linked amelogenesis imperfecta (Lench & Winter 1995). In the future, progress in ongoing molecular, biochemical and histological research will improve our ability to diagnose amelogenesis imperfecta and, as a consequence, the quality of our treatment.

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DISCUSSION

Winter: My unit has published a paper on facial dysplasia and amelogenesis imperfecta which is interesting because it illustrates how a single gene anomaly is not only responsible for an enamel defect but also for an associated structural facial abnormality (Rowley et al 1982). In 44% of our cases the lower face height was outside the normal range. Roughly half of these cases had an anterior open bite (24% of all cases) which was skeletal in type and not due to local factors such as sensitive teeth. Persson & Sundell (1982) have observed similar findings. Therefore, this has major implications not just in enamel development but also in craniofacial development.

Thesleff: It is possible that functional abnormalities explain the open bite deformities in amelogenesis imperfecta. Because teeth are sensitive, the patients tend to bite with a lesser force, and the resulting weak muscle activity may lead to the open bites. One cannot rule out the possibility that the craniofacial abnormality is a secondary consequence to the enamel defects.

Bäckman: The skeletal open bite deformity is also found in cases without dentoalveolar open bites.

Thesleff: But it is difficult to explain that an enamel gene defect could cause craniofacial defects other than that it is the consequence of the abnormal enamel.

Winter: It cannot be functional because the whole of the lower facial skeleton is abnormal. The cephalometric measurements in affected cases are all outside the normal range.

Thesleff: But the bones of the jaws start to develop very early and the correct function of muscles and other surrounding tissues is essential for normal bone growth. Loss of function produces skeletal changes in the craniofacial bones, and particularly open bites.

Winter: In hypoplastic amelogenesis imperfecta, the primary dentition is not severely affected and the dentition functions quite normally. I can't believe that the facial vertical dysgnathia is due to a disturbance of function. We're talking about early changes because some of these children were as young as six years of age and their permanent dentition had not developed significantly.

Wright: Persson & Sundell (1982) identified the association between open bites and amelogenesis imperfecta. They also concluded that there was no correlation between hypersensitive dentition resulting in a lack of function and open bites, which was why they said it was a pleiotropic effect. They found that in cases where there was normal function and no sensitivity, the skeletal effects were still observed. From this they concluded that the skeletal open bites frequently associated with amelogenesis imperfecta are likely a pleiotropic effect of the gene mutation and not a functional change in mastication or posturing.

Deutsch: I have two questions. First, how sensitive is the linkage analysis, i.e. is it possible to detect a false effect? And second, if two families have the same symptoms do they have to have the same mutation?

Bäckman: Linkage analysis is not an overly sensitive method. It depends on the power of the family material. False positive data can be obtained when there are too few cases to examine and when the available markers are not informative on the actual parts of the chromosome. Therefore, linkage analysis requires large, well-defined families and should include as many cases as possible.

In answer to your second question, they do not necessarily have to have the same mutation. Also, if two individuals have the same mutation they will not necessarily have the same clinical phenotype.

Snead: Just because these children have amelogenesis imperfecta doesn't mean to say that they don't have other problems. They may have other growth factor deficiencies or other types of genetic problems. You said that you had some weak linkage, and I assume this represents a LOD score of about two, but does this linkage improve if you change the recombination value?

Bäckman: These data were obtained by two-point analysis, so the recombination value cannot be changed.

Aldred: The way this is done is to use the most polymorphic markers, and if they're all fully informative then the only factor that limits the LOD score is the number of meioses you're looking at. Therefore, if there are only four people in a family you are limited unless you can combine families and predict that they have mutations at the same genetic locus.

Yamada: You showed a 9 bp deletion in exon 2. What is this mutation?

Bäckman: Three amino acids are lost and one is exchanged in the signal peptide.

Slavkin: The degree of hypocalcification in X-linked amelogenesis imperfecta is at most 58–60%. It is possible that the Y amelogenin gene compensates for the loss of the X amelogenin gene, so that it becomes responsible for retaining roughly 50% of the mineral. Have you looked at expression of the Y amelogenin gene in these X-linked cases?

Bäckman: No.

Aldred: One of the problems is that, for ethical reasons, we have not had the opportunity to remove developing teeth from the boys.

Simmer: One would assume that in two cases of X-linked amelogenesis imperfecta generated by separate defects in the X chromosomal copy of the amelogenin gene there would be no differences in the expression of the Y chromosomal copy. Therefore, it could rescue the X gene in one but not the other. In one case the amelogenin protein may be totally eliminated and in another the amelogenin may be synthesized but not able to be secreted. The latter scenario may cause damage to the ameloblasts and result in a more severe phenotype.

Veis: Why are the primary teeth less affected than the permanent teeth?

Bäckman: It could be related to the shorter mineralization period.

Winter: I have also observed that the lingual surface of teeth are often less affected than the buccal surfaces.

Boyd: These two observations are related because deciduous teeth and the lingual surface of permanent teeth (at least the incisors) are both areas with thinner enamel.

Snead: From a developmental standpoint, hypoplastic amelogenesis imperfecta has linear bands, whereas X-linked amelogenesis imperfecta, although clearly lyonized and mosaic, has vertical bands. Thinking about the ameloblast decussation angle and considering ameloblast clonality, why do we observe such strikingly different morphologies?

Thesleff: The horizontal bands are probably due to the timing of the defects, and the vertical bands are due to lyonization at a very early stage.

Aldred: The margins between ridges and grooves, whether vertical or horizontal, tend to be smooth rather than angular because of the decussation of the enamel prisms. We also have some evidence that the lyonization phenomenon may not be random (Aldred et al 1995).

Snead: But Alan Boyd has demonstrated that the decussation angle determined the angle of wear, and ridges were formed in places of wear (Boyde 1997, this volume).

Boyde: That was for vertical decussation. I was also thinking during the presentation that it would be a wonderful experimental system to study what happens to decussation when there isn't any enamel to decussate in different directions.

Snead: If one assumes that differentiation initiates at the tops of the cusps and works its way down, and if there is a horizontal defect at the top, how can another horizontal defect occur towards the dentine-enamel junction if the precursors are wiped out?

Aldred: In the case of horizontal grooves, I can only speculate that a genetic signal must produce those grooves and something disables those cells for a period of time. We have some evidence in one of our X-linked cases for a temporospatial abnormality: there are porous holes across the tooth at right angles to the vertical markings typical of X-linked amelogenesis imperfecta.

Snead: Could this be an attempt at repair?

Aldred: It could be repair or every now and then they could become more susceptible to environmental changes so that they produce enamel which isn't quite as normal as it should be. We can only speculate because we don't know which genes are dysfunctional.

Wright: It seemed to me that the linear banding pattern was continuous at the same developmental stage on the teeth regardless of their chronology. Thus, an incisor had a hypoplastic band at the same height as a premolar even though these teeth form at very different time periods. The same effect occurs in the snow-capped teeth, where the hypomineralized defect is distributed on the coronal area of all the teeth despite the difference in developmental timing. This suggests that a genetic programme at a specific point in time causes the malfunction.

Slavkin: Does a similar situation occur with tetracycline?

Wright: No, because tetracycline would label each tooth at the specific point that they're developing, and all teeth are developing differently. Tetracycline given to a two year old would label the middle of the permanent, central incisors and not the premolars at all. In contrast, we observe that in these cases of amelogenesis imperfecta, a third molar that develops at age 16 years has a band at the same point on the crown as an incisor that develops at age one year.

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Regulation of amelogenin gene expression

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Abstract. The amelogenins are found uniquely in enamel, where they constitute the predominant class of secreted matrix proteins and where they play a fundamental role in normal enamel formation. To better understand the high level of tissue-specific expression, we cloned the bovine X and Y chromosomal amelogenin genes and the murine amelogenin gene and determined the DNA sequences for the regions upstream of the transcription start sites. We observed segments of strong homology among species, and identified consensus sequences for the binding of various transcription factors, including the glucocorticoid receptor, AP1, RXR and p53. Although specific *cis*-elements conferring enhanced transcription have not yet been identified, elements have been localized that have a silencing effect in non-ameloblast cells. Conserved sequences are likely to be involved in tissue-specific expression. Transgenic mouse studies have shown that 3.5 kb of upstream region is sufficient but 900 bp is insufficient for specific expression *in vivo*. Alternative splicing of the primary transcript is an effective mechanism for generating molecular heterogeneity. Amelogenin genes contain seven exons, and exons 3, 4, 5 and most of 6 can be deleted by alternative splicing. However, the pattern of exon splicing varies according to the species, and skipping of bovine exon 3 appears to be developmentally regulated. It will be important to determine whether the relative amounts of translation products differ among species as do the mRNAs, and to correlate the various protein structures with function. These findings also suggest that the regulation of amelogenin gene expression is complex and takes place at several levels.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 187-199

During the development of tooth enamel, ameloblasts secrete an organic matrix composed primarily of amelogenins and various non-amelogenin proteins, such as enamelines and tuft proteins (Eastoe 1979, Deutsch 1989, Limeback 1991, Brookes et al 1995). Amelogenins constitute approximately 90% of the enamel matrix protein (Termine et al 1980) and are thought to be crucial for proper enamel structure and function, since mutations within the X-chromosomal amelogenin gene are strongly

¹This paper was presented by J. Rosenbloom.

correlated with the inherited enamel disease, amelogenesis imperfecta (Lagerström et al 1991, Aldred et al 1992, Lench & Winter 1995, Collier et al 1996). Furthermore, inhibition of amelogenin expression in cultured tooth explants resulted in disorganized enamel containing smaller than normal hydroxyapatite crystals, supporting the theory that amelogenins have an important role in the development of enamel mineral (Diekwisch et al 1993).

Active amelogenin genes are located on the human and bovine X and Y chromosomes, but solely on the X chromosome in the mouse. Expression of amelogenin genes seems to be enamel specific, as expression has not been reported in other mineralizing or non-mineralizing tissues. In order to better understand the regulation of tissue-specific expression, we have cloned the upstream regions of various amelogenin genes and compared the DNA sequences, which has revealed significant homology between species. A 3.5 kb segment of the bovine X-chromosomal amelogenin gene upstream region is able to direct expression of the β -galactosidase reporter gene in ameloblasts of transgenic mice, which suggests that the tissue-specific elements reside in this region. The *cis*-elements conferring enhanced transcription have not yet been identified, but two fragments of the upstream region have been identified that have a silencing effect in non-ameloblast cells (Adeleke-Stainback et al 1995). The conserved sequences are likely to be involved in the stringent tissue-specific expression of this gene.

The two most abundant bovine X-chromosomal amelogenin mRNAs are approximately 850 and 450 nt long. The 850 nt mRNA encodes the 197 amino acid amelogenin protein and the predominant small mRNA encodes the leucine-rich amelogenin protein (LRAP) (Fincham et al 1983, Gibson et al 1991a). The relatively abundant 59 amino acid LRAP is translated from an mRNA in which an alternative RNA splice site within exon 6 is activated, which deletes a large segment of the exon whilst maintaining the reading frame. As three additional X-chromosomal and two Y-chromosomal bovine amelogenin messages have been described (Gibson et al 1991b, 1992, 1995), it is concluded that alternative splicing and sexual dimorphism are both likely to contribute to the heterogeneity of amelogenin proteins.

Methods

Isolation of the murine amelogenin gene. We probed a plated LambdaFixII murine genomic library with an *Eco*RI/*Hind*III fragment of bovine amelogenin cDNA (Gibson et al 1991b). Three plaques remained positive following the tertiary screen, and to date one has been analysed by Southern blot, subcloning and DNA sequence analysis.

DNA sequence determination and analysis. We determined the DNA sequence as described by Chen et al (1994) and analysed the upstream region of the various amelogenin genes using the Compare program (Genetics Computer Group Inc., Madison, WI) and homology dot plots. We compared the bovine X-chromosomal amelogenin upstream region with the transcription factor database Findpatterns (Genetics Computer Group

Inc., Madison, WI) to determine the location of consensus sequences for potential factor binding.

Structure of the amelogenin genes

We isolated the murine amelogenin gene from a genomic library by screening with a 640 bp fragment of bovine amelogenin cDNA and characterized the structure by Southern blotting. Clone 3-3-A contains the upstream region, exons 1–5 and at least part of exon 6. The bovine X and Y chromosomal genes, including the upstream regions, have been cloned previously (Gibson et al 1991b, 1992, Chen et al 1994).

In the human cDNA cloning experiments, one clone contained an additional sequence referred to as exon 4 (Salido et al 1992). A similar region was subsequently identified in murine cDNA and porcine protein (Simmer et al 1994, Yamakoshi et al 1994). Further analysis of the bovine X-chromosomal gene to determine whether an exon similar to exon 4 was present indicated that such a sequence could be found within the intron downstream from exon 3. We synthesized PCR primers in order to determine whether this exon is included within amelogenin mRNA. Reverse transcriptase PCR with the exon 4 primer and a downstream primer amplified a single product containing exon 4, exon 5 and the 3' end of exon 6 (using the splice junction found in LRAP), revealing that exon 4 can be part of the transcribed mRNA. Therefore, the bovine exons were renumbered 1–7, in agreement with the human and murine genes (Yuan et al 1996). The bovine, murine and human amelogenin genes have the general structure shown in Fig. 1. The Y-chromosomal genes have a similar structure; however, it is not known whether exon 4 is present.

Only a few genes have been described that are active on both X and Y chromosomes. In the case of bovine amelogenins, sexual dimorphism will lead to amelogenins with differences in sequences. A significant difference between bovine X- and Y-chromosomal cDNAs is a 63 nt region present in the X-chromosomal, but absent from the Y-chromosomal exon 6 (Gibson et al 1992). There are 41 additional nucleotide differences, resulting in 15 different amino acids in the translated protein.

Characterization of the amelogenin gene promoter

DNA sequence analysis. In order to better understand regulation of expression of the amelogenin genes, we are currently sequencing the murine promoter and upstream regions. The DNA sequence of 3.5 kb of the bovine X-chromosomal gene and 1.1 kb of the Y-chromosomal gene have been determined. So far, we have sequenced 250 bp of the murine upstream region.

Consensus sequences for transcription factor binding. To search for regions of similarity, we compared the bovine X-chromosomal upstream region to the bovine Y and murine upstream regions, and presented the results as a dot plot. In the case where there is significant homology, the dots align on the diagonal (Fig. 2). Significant regions of

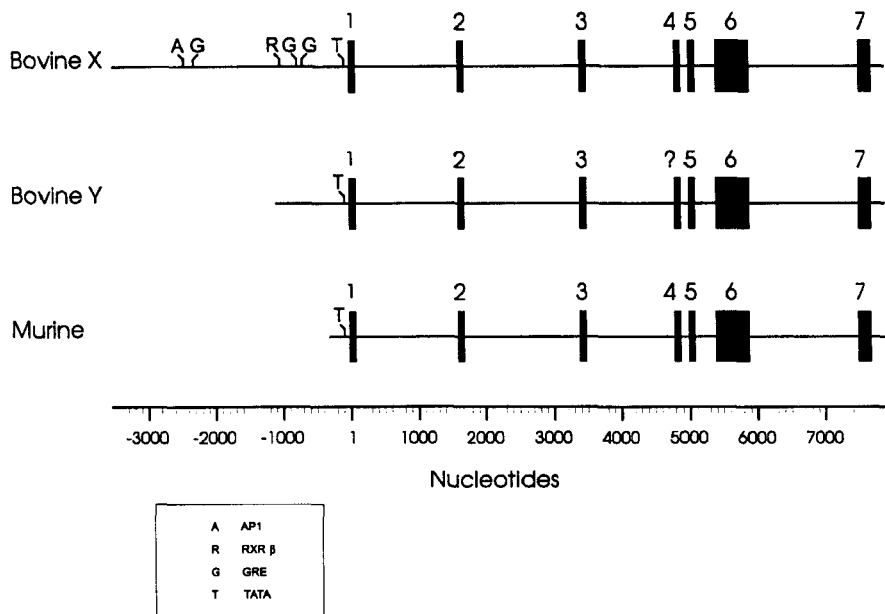


FIG. 1. Diagrammatic representation of the bovine and mouse amelogenin genes. Bovine exons of X-chromosomal genes have been renumbered following the identification of exon 4, which has not yet been found in Y-chromosomal genes. The length of the upstream regions that have been sequenced is indicated, and the location of consensus sequences for transcription factor binding and the TATA box are shown.

homology with the human DNA can also be observed (not shown). Transcription factor-binding consensus sequences were searched for using the program Findpatterns (Fig. 1), which shows potential sites for AP1, RXR β , and glucocorticoid receptor binding, as well as the TATA box in the bovine X-chromosomal gene. In addition there are nine consensus sequences for binding of p53 scattered throughout the sequence. The Y-chromosomal and murine gene upstream regions are currently undergoing analysis.

Expression of the amelogenin gene

Expression in transgenic mice. Primer extension experiments had determined the position of transcription initiation on the X-chromosomal gene, which is the upstream boundary of exon 1 (Fig. 1). We constructed a transgenic mouse using an expression vector containing 3.5 kb of the upstream region ending within exon 1 that was capable of driving expression of the β -galactosidase gene. We dissected mandibles from one- to four-day-old mice and stained them with the substrate X-gal. We observed a blue precipitate in ameloblasts and occasionally in the adjacent stratum intermedium

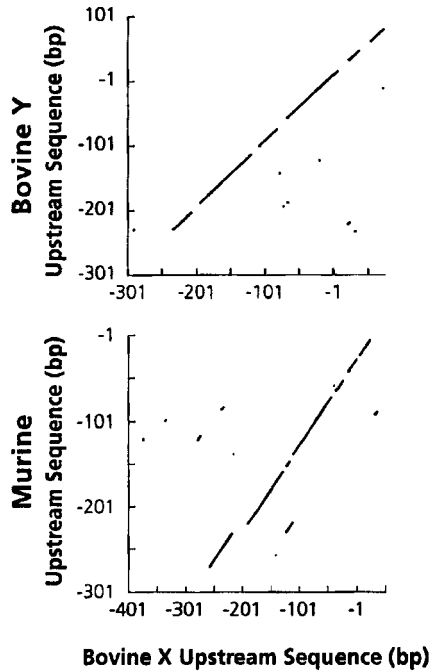


FIG. 2. Comparison of the bovine X-chromosomal amelogenin gene upstream region to the Y-chromosomal and murine genes by dot plot. Homologous sequences align on the diagonal.

cells (Chen et al 1994, Adeleke-Stainback et al 1995). A vector that contained 900 bp of upstream region, also ending within exon 1, did not direct detectable expression, indicating that 3.5 kb is sufficient and 900 bp insufficient to direct reporter gene expression properly to ameloblasts.

Identification of silencing elements. We initially searched for elements required for the stringent tissue-specific regulation of this gene by searching for upstream regions able to reduce expression of the heterologous SV40 promoter in non-ameloblast cells (Adeleke-Stainback et al 1995). We identified two fragments, 1.5 and 1.9 kb in length, that reduced SV40-directed chloramphenicol acetyltransferase expression in HeLa cells (Table 1). Experiments are currently underway to specifically identify the location of elements with activity within these regions, using the heterologous thymidine kinase promoter (Park et al 1994).

Heterogeneity of amelogenin mRNA

X- and Y-chromosomal amelogenin mRNAs. RNAs transcribed from both X- and Y-chromosomal amelogenin genes are subject to alternative splicing. The structures of the various amelogenin mRNAs are shown in Fig. 3, and the information currently

TABLE 1 Amelogenin gene fragments with silencing activity in HeLa cells

<i>Plasmid^a</i>	<i>Description</i>	<i>Per cent of control</i>
pCAT promoter	SV40 promoter, no insert	5.70
pCATBAX1.9	SV40 promoter+1.9 kb upstream (– 3500 to – 1550)	2.44
pCATBAX1.5	SV40 promoter+1.5 kb upstream (– 1550 to – 50)	1.90
pCATbasic	No promoter or insert	1.52

^aChloramphenicol acetyltransferase (CAT) plasmids co-transfected into HeLa cells with the β -galactosidase expression vector pCH110 for normalization. CAT assays performed 48 h after transfection as described by Adeleke-Stainback et al (1995). Positive control: pCAT control containing SV40 promoter and enhancer.

available for expression of the various alternatively spliced bovine mRNAs is given in Table 2. mRNAs that encode BX197 and BX59 are known to be translated; however, each of the mRNAs may be translated at some level. The X-chromosomal gene has greater transcriptional activity compared to the Y-chromosomal gene (Salido et al 1992, Yuan et al 1996), because six to 10 times more X-chromosomal mRNA is present in developing teeth (Fig. 4). This measurement is possible in cows because the 3' untranslated regions of the mRNAs are sufficiently divergent to allow construction of specific probes for Northern blots. Y-chromosomal mRNA is not seen in RNA from female animals (lane 3), and since the consensus sequence AG for splicing within X-chromosomal amelogenin exon 6 is replaced by AA in the Y-chromosomal gene, a 450 nt band is not seen when the Y-specific probe is used (lanes 3 and 4).

Species-specific and developmental regulation of alternative splicing. There are several differences between the various alternatively spliced mRNAs that have been identified in different species. Exon 3 is reported to be invariably present in the mouse, but it can be spliced from human, bovine and porcine message (Salido et al 1992, Simmer et al 1994, Yuan et al 1996, Yamakoshi et al 1994), and the presence of exon 3 appears to be developmentally regulated in cows (Yuan et al 1996, Table 3). We probed the Northern blot in Fig. 5A with an oligomer that spans exons 2 and 5 to identify the mRNA that encodes BX181. As exon 3 contains the sole site for amelogenin protein phosphorylation (Fincham & Moradian-Oldak 1993), its absence from some amelogenins may provide them with a somewhat different function. Interestingly, exon 5 is invariably present in bovine message, but can be spliced from murine mRNA (Simmer et al 1994), and murine exon 6 has several internal splice sites.

Amelogenin proteins are highly conserved across species, but exon 4 is a conspicuous exception, as there is little similarity between species (Fig. 6). In addition, amelogenin including exon 4 appears to be relatively abundant in mouse, human and hamster cDNAs, whereas it is rarely found in bovine cDNA (Simmer et al 1994, Yuan et al 1996).

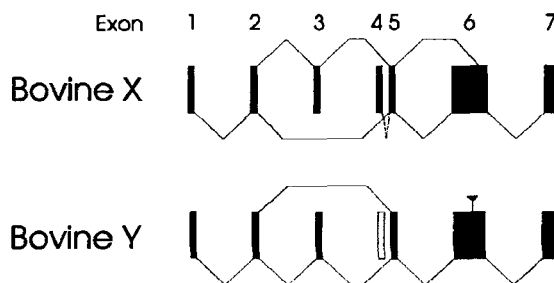


FIG. 3. Diagram of alternative splicing for bovine X- and Y- chromosomal amelogenin messages. The small black triangle represents 63 bp found only in exon 6 of the X-chromosomal gene.

LRAP is present in murine, bovine and porcine amelogenins, and it is translated at a relatively high level. Therefore, the peptide has been isolated and identified by amino acid sequencing (Fincham et al 1983, Yamakoshi et al 1994). In addition, the rat LRAP cDNA has been cloned and sequenced (Bonass et al 1994). We probed the Northern blot in Fig. 5B with a labelled oligomer that spans the LRAP splice junction between exon 5 and the internal exon 6 site to identify the mRNA that encodes BX59. The

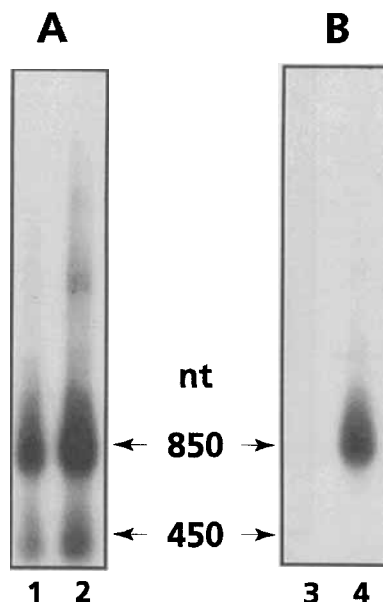


FIG. 4. Northern blots of bovine enamel organ total RNA. Probed for X-chromosomal (A) or Y-chromosomal (B) amelogenin mRNA. Lanes 1 and 3, RNA from a Fetal Day 182 female; lanes 2 and 4, RNA from a Fetal Day 184 male.

TABLE 2 Bovine amelogenin cDNAs

<i>cDNA^a</i>	<i>Exons included</i>	<i>Comments</i>
BX197	1, 2, 3, 5, 6, 7	Predominant amelogenin
BX181	1, 2, 5, 6, 7	Developmentally regulated
BX59	1, 2, 3, 5, 3'6, 7	Leucine-rich amelogenin polypeptide
BX43	1, 2, 5, 3'6, 7	Small amelogenin peptide, rare
BX ^b	?, 4, 5, 3'6, 7	Rare
BY176	1, 2, 3, 5, 6, 7	Male specific
BY160	1, 2, 5, 6, 7	Male specific, rare

^aThe numbers in the cDNA designations correspond to the number of amino acid residues encoded by the cDNA.

^bClones containing exon 4 also contain intron 4, which includes an in-frame translation termination codon. The 5' end has not been determined.

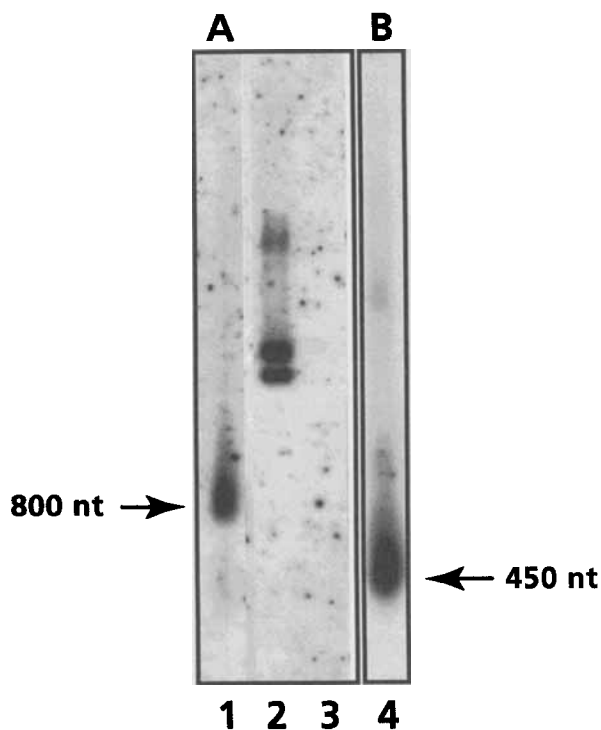


FIG. 5. Northern blots of bovine enamel organ total RNA probed for mRNA from which exons 3 and 4 were deleted (A) or for the leucine-rich amelogenin protein mRNA (B). Lanes 1 and 4, RNA from a Fetal Day 140 male; lane 2, plasmid positive control, containing exons 2 and 5; lane 3, plasmid negative control, containing exons 2, 3 and 5.

TABLE 3 Ratios of alternatively spliced mRNAs

<i>mRNA</i> ^a	140 days	156 days	158 days	179 days	200 days
LRAP	0.32	0.27	0.25	0.31	0.29
Lacking exon 3 and 4	0.75	0.74	0.67	0.46	0.33

^aNorthern blots containing RNA from fetal teeth of cows at various ages probed with labelled oligomers specific for the leucine-rich amelogenin protein (LRAP) or the exon 2 to 5 splice junctions. Blots reprobed for total X-chromosomal amelogenin message and ratios calculated following densitometry.

relative amount of LRAP message in bovine mandibular molars does not change appreciably between 4.5 and 6.5 months of fetal age (Yuan et al 1996, Table 3).

Conclusion

Regulation of expression of the amelogenin genes appears to be at the level of transcriptional control of sexually dimorphic genes, whereas alternative splicing contributes to the heterogeneity of translated protein products. Even in the mouse, which has only one active gene, at least eight alternatively spliced mRNAs have been identified. It is interesting to note that four bovine amelogenin exons are either much smaller or much larger than the average exon size of 137 nt (Hawkins 1988). For example, exon 4 contains only 42 nt, and exons shorter than 50 nt are frequently deleted by alternative splicing (Berget 1995). Exon 6, which is 489 nt, is longer than 99% of primate internal exons, and this exon in the amelogenin transcript is subject to substantial internal splicing. Perhaps the amelogenin genes have evolved in such a way as to promote this extensive alternative splicing. Understanding the reason for the differences in the relative amounts of various amelogenins found in different species and during development is likely to lead to enhanced insight regarding the function of the various amelogenins during enamel mineral formation.

Bovine X	N S Y F Q G I S I D K T A L V S L Y F M Q L N
Human	N S H S Q A I N V D R T A L
Murine	K S H S Q A I N T D R T A L
Porcine	K S G S W G A X L T A F V S Y V Q

FIG. 6. Predicted amino acid sequence of amelogenin exon 4. Porcine x and y are thought to be arginine and proline, respectively (Yamakoshi et al 1994, Salido et al 1992, Simmer et al 1994, Yuan et al 1996).

Acknowledgements

We would like to thank M. Young for the murine genomic library, the Biopolymer Analysis Laboratory for DNA sequence determination and W. Abrams for assistance with the figures. Support for this research was provided from grants DE 09164, DE 10149 and DE 11089 (C. W. G.) and DE 08239 (J. R.) from the National Institute of Dental Research, National Institutes of Health.

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DISCUSSION

Yamada: There is a striking conservation between the promoter regions of the amelogenin gene in different species. We are trying to identify the promoter region of the ameloblastin gene. We may be able to pin-point the regulatory sequences for tissue-specific expression by comparing the promoters of these different genes. You mentioned that there is a silencer element in the promoter region. Have you done any experiments to delineate a sequence for silencer activity?

Rosenbloom: We are in the process of doing that. So far, we have narrowed the two segments down to about 300 bp, but I expect that the region will turn out to be smaller than that.

Zeichner-David: What system did you use to test for the silencer element?

Rosenbloom: We used either a heterologous SV40 promoter or a heterologous thymidine kinase reporter to drive the expression of the reporter gene containing the test segment, which can be placed either downstream or upstream of the promoter.

Butler: It is my understanding that there are no cells with which to perform transfection assays, but you mentioned that you have tried these experiments. What cells are you using?

Rosenbloom: Immortalized cells maintain a differentiated function, so we are trying to culture ameloblasts from *p53* knockout mice, and we are also looking at SV40 T antigen transgenic mice, but we are having some difficulties with this.

Yamada: We have used an adenovirus vector system in which we can introduce an expression plasmid. Infection of cells with virus solution containing the plasmid allows the plasmid to internalize into cells in organ culture. Another approach is to analyse the

promoter activity in heterozygous transgenic mice. This is quicker than analysing it in homozygous transgenic mouse lines.

Simmer: C.-C. Hu, M. Fukae, T. Uchida et al (unpublished work 1996) obtained 20 porcine amelogenin cDNA clones and found that 13 of these contain one form of exon 1 and the remaining seven contain another. Exons 3–6 are identical in all cases. This suggests that there are two different promoters in the porcine amelogenin gene. Have you looked at the 5' end of the amelogenin gene to see if there are multiple promoters in the bovine amelogenin gene?

Rosenbloom: We haven't looked.

Simmer: In your primer extension studies, from which exon did you prime DNA synthesis?

Rosenbloom: Exon 2.

Simmer: The porcine gene has two different forms of exon 2 and both are always paired with their own exon 1.

Rosenbloom: But we wouldn't have detected this.

Snead: We have encountered the same problem that Joel Rosenbloom is addressing, but we have addressed it in a slightly different way. Although we have mapped the X and Y amelogenin genes in mice and humans, we decided that studying the mouse genes would be simpler because they only have one X chromosome copy and we wouldn't have to worry about Y-chromosomal compensation. We identified a 2200 bp amelogenin promoter which we placed into a construct that drives the expression of the luciferase gene (Snead et al 1996). We found that the expression pattern of the luciferase construct along the major buccal cusp of the first mandibular molar in newborn mice was identical to the expression pattern of amelogenin. We then used a defective retrovirus containing a temperature-sensitive large T antigen to immortalize enamel organ epithelia so that we could produce an ameloblast-like cell line which would enable us to identify regulatory regions within the 2200 bp promoter. This retrovirus is essentially 100% efficient at transfecting DNA into primary enamel organ epithelial cells, so it has been possible to obtain cells that express amelogenin at reasonably high levels. By making various deletions in the promoter construct, we found that the region containing the first 400 nucleotides gives approximately the same level of expression as the full-length amelogenin sequence in either primary enamel organ epithelial cells or large T antigen-expressing enamel organ epithelial cells. We are now trying to identify the *in vivo* transcription factors that bind to this regulatory element.

In related studies, our results from the two-hybrid system indicate that specific domains in amelogenin are required for enamel matrix assembly, so we would also like to produce dominant negative mutations in those domains, hook them up to the amelogenin promoter and make transgenic mice. We hope that this strategy will generate a dominant negative phenotype, i.e. an amelogenesis imperfecta-like strain.

Yamada: In Joel Rosenbloom's transgenic mice, expression of amelogenin was not detected before birth. Have you detected expression before birth?

Snead: We found that the expression is less robust at earlier stages. The luciferase system is considerably more sensitive than using galactosidase, which may explain the differences. Also, we injected many copies of the construct into the pronucleus so that these mice have various copy numbers of the transgene. Therefore, there will be an excess of the amelogenin promoter, which may have an effect on regulation because there are only limited amounts of transcription factors. It is not surprising that there may be slight differences between transgenic and normal mice.

Slavkin: Reverse transcription (RT) PCR analyses have also detected amelogenin mRNA at the early cap stage, which is in agreement with your luciferase results.

Butler: RT-PCR analysis may be too sensitive to detect what's really happening because it can detect even the lowest levels of transcript and may thus provide irrelevant data.

Zeichner-David: It tells you that the cells are expressing the mRNA. Just because they are doing so at a low level does not mean that it is not significant.

Slavkin: It is significant if you are addressing what enables a cell to actually switch on expression; whereas it may not be significant if you are looking at the activation of a protein that is regulated by translation or secretion.

Snead: The reason why we did that experiment was because we were interested in deciphering the instructive signals as opposed to the permissive signals (Couwenhoven & Snead 1994). We wanted to determine the developmental time at which the enamel epithelium is committed to differentiate into the amelogenin-expressing phenotype when put into a permissive environment.

Zeichner-David: Can you tell us a bit more about the induction experiments you performed in this work?

Snead: We separated the epithelium from the mesenchyme of developing mouse molars (we checked that the epithelium was free of mesenchyme by testing for mesenchyme-specific cytoskeletal elements using RT-PCR). We cultured the epithelial cells in matrigel, recapitulating time at about two days *in vitro* for every one day *in vivo*. We found that only enamel organ epithelium equivalent to the 15th day of gestation and thereafter, when recombined into a non-cellular, non-signalling matrix, is capable of supporting ameloblast differentiation, i.e. with respect to the identification of amelogenin mRNA.

Zeichner-David: In other words, amelogenin is not expressed prior to the 15th day of gestation. However, tuftelin is already being expressed at this stage, suggesting that there are two separate regulators.

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Molecular biology of hereditary enamel defects

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Abstract. Amelogenesis imperfecta is a disfiguring inherited condition affecting tooth enamel. X-Linked and autosomal dominant and recessive inheritance patterns occur. X-Linked amelogenesis imperfecta has been studied extensively at the molecular level. Linkage analysis has shown that there is genetic heterogeneity in X-linked amelogenesis imperfecta with two identified loci: AIH1 and AIH3. The AIH1 locus corresponds to the location of the amelogenin gene on the distal short arm of the X chromosome; various mutations in the amelogenin gene have been found in families with X-linked amelogenesis imperfecta. The AIH3 locus maps to the Xq24-q27.1 region on the long arm of the X chromosome. Linkage to the long arm of chromosome 4 has been established in three families with autosomal dominant amelogenesis imperfecta. There is as yet no published evidence for genetic heterogeneity in autosomal dominant amelogenesis imperfecta as in X-linked amelogenesis imperfecta. Candidate genes for autosomal dominant amelogenesis imperfecta include tuftelin (1q), albumin (4q) and ameloblastin (4q) but the involvement of these genes in the disease has yet to be demonstrated. In view of the variable clinical appearances within families with autosomal dominant amelogenesis imperfecta and X-linked amelogenesis imperfecta, together with the finding that different X-linked amelogenesis imperfecta phenotypes result from mutations within the same gene, an alternative classification based on the molecular defect and mode of inheritance rather than phenotype has been proposed.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 200-209

This chapter will summarize the current understanding of the molecular biology of inherited enamel defects. Evidence stems from linkage analysis studies of families with amelogenesis imperfecta supplemented by investigations based on the molecular biology of developing enamel.

The 1980s saw the emergence of molecular biology techniques as an aid to the investigation of inherited diseases. Central to this has been the use of linkage analysis, which relies upon the segregation of an inherited trait in a pedigree with linked DNA markers from the adjacent part of the chromosome. Initially, this was accomplished using restriction fragment length polymorphisms, but more recently variable number of tandem repeat (VNTR) markers have been used for the same

purpose; these VNTRs are highly polymorphic and therefore provide a more powerful tool for linkage studies. The identification of families with an X-linked mode of inheritance limits the scope of investigation to a single chromosome (the X chromosome), hence it is not too surprising that the first inherited enamel disorder gene mapped was localized to the X chromosome. The clue to the region of localization of the first locus on the X chromosome causing X-linked amelogenesis imperfecta came from parallel studies of developing enamel proteins. Later studies have led to the finding of genetic heterogeneity in X-linked amelogenesis imperfecta, the identification of a locus for autosomal dominant amelogenesis imperfecta on chromosome 4 and candidate genes for autosomal dominant amelogenesis imperfecta.

X-Linked amelogenesis imperfecta

Linkage analysis studies

In X-linked conditions affected males pass on the disease to all of their daughters but not to their sons (by virtue of males having only a single [mutant] X chromosome), whereas heterozygous females can pass on the trait to children of either sex. Thus, in the latter situation, hemizygous males are fully affected, whereas their heterozygous sisters feature lyonization (Lyon 1961, 1962); i.e. in the case of X-linked amelogenesis imperfecta, a vertical marking (either of colour or contour or both) of the enamel (Crawford & Aldred 1992) is observed.

Two of the Swedish families documented by Bäckman & Holmgren (1988) displayed the characteristics of an X-linked disorder, both clinically and on pedigree analysis. Males were more severely affected than females, and heterozygous females demonstrated lyonization. These families with X-linked amelogenesis imperfecta were studied by linkage analysis in the late 1980s (Lagerström et al 1990). During the same period, the localization of the amelogenin gene to the terminal portion of the short arm of the human X chromosome (as well as the pericentromeric region of the Y chromosome) was reported by Lau et al (1989). The amelogenin gene therefore became a candidate gene for X-linked amelogenesis imperfecta. The linkage analysis study of Lagerström et al (1990) supported this candidate gene hypothesis by providing evidence of linkage of the disease to the same (Xp22.1-p22.3) region of the X chromosome. Using two-point analysis, they reported a combined LOD score of 4.45 for marker DXS85 at a zero recombination fraction. This suggested the localization of the gene to the interval between DXS89 and DXS43.

Subsequent linkage analysis in two unrelated families with X-linked amelogenesis imperfecta in the UK confirmed linkage to this same region, with a peak LOD score on multipoint analysis of 7.30 at 2 cM distal to DXS16. Study of recombination events indicated that AIH1 lies in the Xp22.2-p22.3 region in the interval between DXS143 and DXS85 (Aldred et al 1992a).

Linkage analysis has also shown that there is genetic heterogeneity in X-linked amelogenesis imperfecta because the disease in a further unrelated family in the UK

was unlinked to the AIH1 locus (Aldred et al 1992a). This second locus, designated AIH3, maps to the Xq24-q27.1 region on the long arm of the X chromosome (Aldred et al 1992a, 1995). Recombinations define the localization to the interval between DXS1001 at Xq24 and DXS984, DXS998 and DXS425 in the Xq26-q27.1 region. Multipoint linkage analysis has given a peak LOD score of 3.00 for marker DXS144E at a zero recombination fraction (M. J. Aldred, L.-C. Zhang, P. J. M. Crawford, unpublished results 1995).

Mutation analysis

One of the families with X-linked amelogenesis imperfecta mapping to the distal short arm of the X chromosome described by Lagerström et al (1990) has been investigated further to identify mutations in the amelogenin gene. Nakahori et al (1991) demonstrated the presence of human genomic sequences with homology to the mouse amelogenin cDNA on the X and Y chromosomes and identified three open reading frames in a 2.6 kb sequenced portion of a region of X-Y homology. This human genomic DNA sequence (Nakahori et al 1991) has been used to design oligonucleotide primers for amplification and sequencing of putative exons of the human X chromosome amelogenin gene in this family (Lagerström et al 1991). A 5 kb deletion in the amelogenin gene segregated with the disease, thereby confirming that a mutation in the X chromosome amelogenin gene could cause X-linked amelogenesis imperfecta (Lagerström et al 1991). The human amelogenin X and Y chromosome genes have since been cloned (Salido et al 1992) and a total of seven exons identified, with exons 3, 5 and 6 corresponding to the open reading frames identified by Nakahori et al (1991).

Based on this information, the 5 kb deletion reported by Lagerström et al (1991) removed five of the seven exons. The deletion extended from the second intron to the last exon, leaving only the first two codons for production of protein (Lagerström-Fermér et al 1993). The similarity of the DNA sequences at each end of the deletion led them to suggest that the mutation occurred as a result of an illegitimate recombination.

Additional studies have identified a range of mutations in the amelogenin gene in families with X-linked amelogenesis imperfecta (Aldred et al 1992b, Lench et al 1994, Lagerström-Fermér et al 1995, Lench & Winter 1995). A nonsense mutation in exon 5 of the amelogenin gene resulting from a single base deletion (CCCC→CCC) has been found by direct DNA sequencing (Aldred et al 1992b). This deletion produced a frameshift and introduced a TGA stop codon into the exon. Lench et al (1994) identified the same mutation using single-strand conformational polymorphism (SSCP) analysis and sequencing in another family, which raises the possibility that these two families are, in fact, related.

Yet another mutation has been reported by Lagerström-Fermér et al (1995). This mutation affects the amelogenin signal peptide by virtue of the loss of three amino

acids and substitution of another amino acid. The authors suggested that this would interfere with the translation of protein synthesis.

A series of three different mutations has been identified by Lench & Winter (1995) by SSCP analysis and DNA sequencing. These were a C→T substitution in exon 5, a G→T substitution in exon 6 and another single cytosine deletion in exon 6.

We have identified two novel mutations in families with amelogenesis imperfecta mapped by linkage analysis to the AIH1/amelogenin gene locus (Aldred et al 1992a). Both of these mutations have been found in exon 6: a single cytosine deletion in one of the families and an A→T substitution in the second family. The deletion mutation leads to premature termination of translation due to a frameshift, whereas the substitution mutation results in an amino acid change from histidine to leucine (N. Jamshidi, M. J. Aldred and P. J. M. Crawford, unpublished results 1995).

There is evidence that the Y chromosome amelogenin gene is expressed (Fincham et al 1991), albeit at low levels (Salido et al 1992), but there is no evidence for any inherited enamel defect being due to mutations in this gene.

Autosomal dominant amelogenesis imperfecta

The molecular basis of the autosomal forms of amelogenesis imperfecta is less well understood. A recent paper has established linkage to the long arm of chromosome 4 in three families with the same phenotype of autosomal dominant amelogenesis imperfecta (Forsman et al 1994). The disease mapped to the region close to the localization for dentinogenesis imperfecta. The albumin gene also maps to the 4q region and, given that albumin has been reported to be one of the proteins in developing enamel, is therefore a candidate gene for autosomal dominant amelogenesis imperfecta. No mutations in the albumin gene have yet been reported in autosomal dominant amelogenesis imperfecta, nor is there yet any published evidence for genetic heterogeneity in autosomal dominant amelogenesis imperfecta as there is in X-linked amelogenesis imperfecta. More recently, the ameloblastin (amelin) gene has been mapped to the same region (Krebsbach et al 1996).

Apart from amelogenin and albumin, the other protein in developing enamel that has been characterized to date is tuftelin, hence this must be another candidate gene for amelogenesis imperfecta. The tuftelin gene (Deutsch et al 1991, 1995) has been mapped to chromosome 1q (Deutsch et al 1994) but there is currently no evidence to suggest linkage of autosomal dominant amelogenesis imperfecta to this region. In the future, any genes coding for proteinases involved in the degradation of enamel proteins will also be candidate genes for inherited enamel defects.

Classification of amelogenesis imperfecta

We have previously pointed out the anomalies in categorizing families with X-linked amelogenesis imperfecta (Crawford & Aldred 1992) and have gone on to review the difficulties in classification of amelogenesis imperfecta based on phenotype (Aldred &

Crawford 1995). In view of the variable clinical appearances within families with autosomal dominant or X-linked amelogenesis imperfecta, together with the finding that different X-linked amelogenesis imperfecta phenotypes result from the same mutation within the amelogenin gene, an alternative classification—based on the molecular defect and mode of inheritance rather than phenotype—has been proposed (Aldred & Crawford 1995). We believe that this scheme offers more flexibility than previous classifications by allowing cases of amelogenesis imperfecta to be included in which there is involvement of other body systems.

Summary

Molecular biological studies have added considerably to our understanding of the molecular basis of inherited disorders of enamel. These diseases have serious social consequences in childhood and throughout life, particularly in relation to teasing because of the appearance of the teeth, and they may be associated with extreme thermal sensitivity which affects everyday life.

Affected individuals with amelogenesis imperfecta require life-long treatment, which can be technically difficult as well as a serious financial burden on the individuals and their families. The determination of the molecular basis of amelogenesis imperfecta in individuals and families in the future will help us to understand the variable phenotypes within and between families. Insights will also be gained into the normal processes in enamel formation as well as informing clinical treatment of abnormal enamel. There remains a need for the development of sensitive and specific tests for diagnostic application in equivocal cases, particularly in distinguishing between amelogenesis imperfecta and fluorosis.

Acknowledgements

The authors wish to thank the families they have studied for their willing participation and support. These studies were supported by the Medical Research Council (UK) and the National Health and Medical Research Council of Australia.

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DISCUSSION

Winter: There is no doubt that we need to develop a new classification. We are currently in an intermediate position in which we can only classify some of the cases. However, as clinicians, we have to advise our patients on the likely outcome of their progeny, so it is currently impractical for clinicians to cast aside their phenotypic classifications. We also need to clarify some of the classifications in McKusick's index (McKusick's *Mendelian Inheritance in Man*, accessed via the worldwide web at the Human

Genome Mapping Project resource centre, Hinxton, UK) because some of them are erroneous.

Aldred: But it is useful in that it classifies the cases according to the mode of inheritance, which is probably more important at the clinical level than the phenotypic label.

Wright: Another example where this problem has arisen is in osteogenesis imperfecta. Hundreds of mutations have been described, but the phenotype-based clinical classification is being used to communicate the conditions effectively to the families of patients. Knowledge of these mutations from a research point of view is useful, but this is not necessarily the case from a clinical point of view.

Aldred: Why can't we just call all of them amelogenesis imperfecta, and then add on the mode of inheritance as the first classification, followed by a description of the phenotype.

Brook: When we proposed a classification 21 years ago we had already looked at about 160 cases of amelogenesis imperfecta and found that a group of them couldn't be placed into any of the categories just by taking into account the main phenotype (Winter & Brook 1975). Therefore, we were already aware of the limitations.

Snead: You mentioned the phenotype of patients with the mutation in exon 6 of amelogenin. Is this mutation within the assembly domain? I would predict that removing the amelogenin assembly domain B will result in an matrix assembly defect that may manifest itself as an amelogenesis imperfecta-like phenotype.

Aldred: We have performed a brief analysis on the location of these mutations. There are many mutations in exon 6, but there doesn't seem to be any particular hotspot. It is possible that it has more mutations simply because it is larger.

Snead: You also mentioned that there is a higher incidence of autosomal cases, but one must remember that only a small portion of the entire genome is on the X chromosome, so it's not surprising that the majority of cases map to the other 95% of the genome.

One impact of dental research on other areas of research is in the area of the amelogenin gene and other genes surrounding the pseudo-autosomal region of the X and Y chromosomes. This region influences sex determination and fertility, as well as containing several genetic hotspots on the X chromosome. The molecular biology of the sickle cell anaemias, a discovery process which revolutionized medical studies, arose as a result of the analysis of the structure of the haemoglobin molecule. Michael Aldred's efforts to understand amelogenesis imperfecta at the level of the genetic locus suggests that we may be able to make similar gains in the field of dentistry.

Slavkin: Michael Aldred has been looking at the genomic DNA of the family with X-linked amelogenesis imperfecta that is not associated with the position of the amelogenin gene, rather it is associated with the other arm of the X chromosome. Have you had the opportunity to look at the homozygous 'tabby' mouse, which allegedly has amelogenesis imperfecta-like features, because the affected gene (which is not the amelogenin gene) may provide an insight into the understanding of amelogenesis imperfecta.

Aldred: No, I haven't. I thought that the tabby mouse was a model for X-linked hypohidrotic ectodermal dysplasia rather than amelogenesis imperfecta.

Slavkin: Has anyone identified the gene defect in the tabby mouse?

Thesleff: We are working on the cloning of the homologous gene in humans (anhidrotic ectodermal dysplasia) in collaboration with a Finnish group. However, we have not observed hypoplasias in the tabby mouse.

Snead: Have you tried single-strand conformation polymorphism (SSCP) analysis or RNA mapping to characterize some of the amelogenesis imperfecta cases?

Aldred: I have tried unsuccessfully to use SSCP on a number of occasions but Nick Lench has managed to get it working (Lench & Winter 1995, Lench et al 1994).

Snead: The gene is relatively small so this approach may be useful in terms of screening.

Veis: Is there any significance in the observation that the genes responsible for amelogenesis imperfecta localize to the autosomal region 4q? Because dentinogenesis imperfecta type 1 and 2, dentine matrix protein 1 and dentine phosphophoryn also localize to this region.

Aldred: These genes may form a cluster or they may be regulated independently. This is why I am interested in patients others have referred to at this symposium with dentinogenesis imperfecta who also have an enamel defect, i.e. because it may be a contiguous gene syndrome.

Wright: The variability in the degree of lyonization in the X-linked cases doesn't surprise me if one assumes a random inactivation of the X chromosomes. In a population of heterozygous females, at one end of the spectrum there would be some that look normal, and at the other some would have similar defects as the males.

Aldred: We have some evidence that lyonization is not random, i.e. that what one cell does influences the decision of another cell (Aldred et al 1995). This explains why we don't see many cases where there are extremes across the whole of the dentition.

Winter: I would like to mention tooth discolouration. Although many of these cases are probably due to a change in the transmission of light, in cases of pigmented hypomaturation the teeth discolour markedly with age: they start off a golden yellow colour and become dark brown by the age of 15.

Aldred: This may be due to the uptake of dietary pigments.

Wright: We have observed unerupted teeth that are highly discoloured all the way through. It is difficult to determine the colour of the tooth because of differences in the refraction of light on rough surfaces, thinness and shining through of dentine, pigments that are taken up after eruption, and pigments that are contained within the tooth at the time of eruption.

Whitford: How difficult is it to distinguish between fluorosis and amelogenesis imperfecta?

Aldred: Sometimes it is difficult at the clinical level to distinguish the equivocal cases. We have been presented with family members who allegedly have the genetic disorder, but their teeth look more like they have dental fluorosis. Sometimes, this is

supported by the pedigree analysis, in which case those individuals might not have been able to inherit the mutant gene.

Winter: I have recently published a paper on this problem (Winter 1996). I have looked at over 30 children who all have the hallmarks of fluorosis and who might have been placed in the category of idiopathic fluorosis in the past. However, they have clearly not been exposed to excessive amounts of fluoride. Eighty-seven per cent of these individuals have taurodontism of their molar teeth. As the prevalence in the normal population is about 6–9%, I feel this is another manifestation of the gene defect.

Fejerskov: It is possible that what is occasionally referred to as idiopathic dental fluorosis could be a particular type of amelogenesis imperfecta. However, from a diagnostic point of view, one characteristic should be kept in mind. Namely, that in cases of idiopathic fluorosis there is a different distribution of enamel changes within the mouth. Children exposed to a daily dose of fluoride during tooth formation have a more severe phenotype in pre-molars/second molars and a much less severe phenotype in teeth that are formed early, which is particularly evident in the lower incisors. It's rare to observe substantial fluorosis in lower incisors, so it may be helpful to look at the distributions within the mouth if you are dealing with apparent cases of fluorosis-like enamel changes in a non-epidemic area.

Brook: Clinically, one of the ways of distinguishing between fluoride-induced defects and amelogenesis imperfecta in some cases of excessive ingestion of fluoride from toothpaste or tablets is a marked contrast between the enamel on the deciduous teeth and the permanent teeth.

Boyde: In many herbivorous mammals peritubular dentine forms before the intratubular dentine, represents more than 50% of the tissue, and is more mineralized and wear resistant than the surrounding dentine. When the tooth wears, it creates a micro-roughness to the functional surface, which is useful for dealing with a plant diet (Boyde & Fortelius 1986). Do the fluorotic teeth have a deficiency in peritubular dentine?

Wright: In all the cases that we've looked at, the peritubular dentine and the root cementum appear to be structurally normal. The variation in sensitivity seems to be related to a lack of insulation, either because there is a high enamel water content, low mineral content or because the enamel is very thin. The enamel serves as the primary insulator in teeth that transmits stimuli to the dentine tubules. In amelogenesis imperfecta hypersensitivity appears to be related to the underlying enamel defects.

Sasaki: Is the disturbance of the matrix specific for enamel or for dentine, or does it occur in both?

Aldred: I don't know.

Sasaki: The reason I ask this is that in osteosclerotic mice, although trabecular bone is formed, bone is not resorbed because the osteoclast is disordered. Hypomineralization of the enamel and dysplasia of the dentine occurs when only part of the alveolar bone is present. The cytoplasm of ameloblasts with enamel hypoplasia contains an abnormally high level of lysosomes, few secretory granules and less matrix production. This also occurs in dentine. Matrix dysplasia is also associated with the decreased cell height in odontoblasts.

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General discussion II

Slavkin: It is always important to justify dental research and, therefore, in order to do this, we must look for reasons why it is relevant to other areas of research. For example, there is reasonable evidence which suggests that scales on the exterior of the body evolved into the oral cavity, so that it is really a sensory apparatus. Therefore, the cranial nerves and the nerve growth factor receptors in teeth may not only be relevant to mastication, but also to sensing the environment, i.e. some of these features probably had more significance before the evolution of teeth, but they are highly conserved and remain part of tooth development in mammals.

Snead: Hal Slavkin, I seem to remember that you and Alan Fincham have looked at the resorption of enamel matrices in cultured teeth? Isn't this similar to the experiments that Takahisa Sasaki described in the discussion after Michael Aldred's presentation?

Slavkin: Yes! A few years ago, we designed a set of experiments to test the hypothesis that following enamel matrix fabrication and during biomineralization, enamel proteins are partially degraded and subsequently removed by post-secretory ameloblasts. We further hypothesized that these products diffuse from the post-secretory ameloblasts into the papillary layer and are then moved into the vasculature. If these assumptions are valid, then in mouse cap stage molar tooth explant cultures, using serum-less medium, metabolically labelled enamel proteins are initially deposited within the extracellular matrix and eventually diffuse from the matrix to the vasculature. Since these studies are done in organ culture and without a blood supply, isotopically labelled polypeptides accumulate in the stellate reticulum region of the enamel organ. This is what we observed. The experimental evidence supported the hypothesis.

Curiously, these observations also raise another potentially significant biological set of issues. Enamel proteins do not normally confront immune cells during formation and maturation of enamel (although they are presumably sequestered within enamel), but are they potential immunogens for producing autoantibodies within the individual? Should we revisit the possibility of circulating antibodies directed against enamel immunogens in selected non-syndromic, as well as syndromic, diseases associated with enamel hyperplasia and/or hypocalcification? This could be quite promising.

Zeichner-David: We have experienced this but only in a few rabbits, so it is unlikely that it involves a normal physiological activity.

Nanci: Indeed, a portion of enamel proteins are secreted laterally between ameloblasts — in rats this can be as much as 10–15%. Although these may be taken

up for degradation by ameloblasts, some of them may also diffuse out of the enamel organ (see Nanci & Smith 1992).

Slavkin: To my knowledge, no one has observed crystals growing in the material that is secreted laterally. Crystal growth seems to be related to an epithelial–mesenchymal interface and is not intrinsic to the distribution of enamel proteins in the fluid between cells.

Nanci: Throughout our studies I have never seen any morphological evidence for the presence of crystals within the patches of enamel proteins found basolaterally along ameloblasts. When we first reported the presence of enamel proteins between ameloblasts one important question was whether all the proteins necessary for crystal formation are present within the patches (Nanci et al 1985). We are interested in three other questions: whether enzymes are necessary for the extracellular processing of enamel proteins, whether these enzymes can function in the interstitial fluid environment and whether the calcium concentration in the patches is sufficient for mineral deposition.

Simmer: Takano et al (1995) showed that large numbers of antigen-presenting cells can be found in the enamel organ. We have observed that anti-amelogenin antibodies raised in rabbits do not recognize rabbit amelogenins. We conclude from these observations that enamel proteins escape through the ameloblast layer, are taken up by antigen-presenting cells and are carried back to the thymus, resulting in the clonal deletion of lymphocytes capable of reacting to enamel proteins.

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Environmental causes of enamel defects

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Abstract. A large number of causes of enamel defects, both environmental and genetic, have been described. However, many of these are derived from case histories and studies of individual conditions. What is needed now is a systematic investigation of the problem. The first requirement in exploring the aetiology further is the standardization of both the clinical diagnosis and the descriptive terminology. This has been provided by the Fédération Dentaire Internationale Developmental Defects of Enamel Index. Comparing studies using standardized methods, including this index, has highlighted areas for closer investigation. The total prevalence of enamel defects in a population needs to be established as a baseline for studies on aetiology. Sixty-eight per cent of 1518 school children in London have enamel defects in the permanent dentition, with 10.5% having 10 or more teeth affected and 14.6% having hypoplasia, i.e. missing enamel. These findings are in contrast to the 37% with hypoplasia found in a group of third to fifth century Romano-Britons from Dorset, England, suggesting further consideration of possible environmental and genetic differences between the two populations. An overall long-term study of dental development in low birth weight children has shown significantly more ($P < 0.001$) enamel defects related to major health problems during the neonatal period. By using standardized, reproducible criteria in prevalence studies to gain an overview of the problem and then studying specific groups or conditions, it is possible to identify general and specific factors in the aetiology of enamel defects and investigate further the varying role of genetic and environmental effects.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 212-225

Developmental defects of enamel present a wide range of clinical features. The defects may affect a circumscribed area of one surface of the enamel or, at the other extreme, they may be widespread, affecting all surfaces of the enamel throughout its full thickness. Similarly, the condition may be localized, involving only one or two teeth, or generalized, affecting many teeth or even the whole dentition. The distribution of the defects may be symmetrical or asymmetrical across the midline of the dentition.

Variation in the clinical appearance also reflects the type and nature of the enamel defect. There may be a full thickness of enamel but a difference from the normal appearance and texture of the surface, or there may be an altered translucency resulting from an area of deficient mineralization. Pitting, grooving or an area of missing enamel may represent deficiencies in the amount of enamel formed. Intrinsic

discolouration gives rise to an altered colour of the teeth. These different types of defect may be present together.

A large number of causes have been described for enamel defects, both environmental and genetic. These may be prenatal, neonatal or postnatal influences on development, and they include trauma, infection, hypoxia and toxins (Small & Murray 1978). However, many specific examples in such a list are derived from studies of case histories and individual conditions. Moreover, rather than a particular appearance being related to a specific cause, the nature and extent of the defects are due to factors such as the severity and duration of the insult, the developmental stage and the response to the injury (Brook & Winter 1975).

Problems arise in attempting to compare findings published in the literature because different terminologies and definitions have been used, together with a variety of indices (Dean 1934, Moller 1962, Young 1973, Thylstrup & Fejerskov 1978, Murray & Shaw 1979, Horowitz et al 1984). Thus, the differences for the prevalence of enamel defects between some studies of low birthweight, pre-term infants (Grahnen et al 1974, Johnsen et al 1984, Soew et al 1987) may be due to varying diagnostic criteria rather than true differences between the groups examined.

Therefore, in studying the aetiology of enamel defects there is a need for a standardized system of examination, with defined terminology and criteria for diagnosis, examination conditions, methodology and recording of data. The index used should not make a presumptive diagnosis of aetiology from the clinical appearance (Cutress et al 1985). This is reinforced by the findings of Winter (1996) who records a number of patients whose clinical appearance is compatible with that previously described as 'fluorosis' but in whom there are well-established histories of the condition in different generations.

A standardized system of examination

The Fédération Dentaire Internationale Developmental Defects of Enamel (FDI DDE) Index (Fédération Dentaire Internationale 1982) is outlined below.

(1) *Type of defect*

normal

opacity: white/cream or yellow/brown

hypoplasia: pits, horizontal grooves, vertical grooves or missing enamel

discoloured enamel not associated with opacity

other defects

combination of defects

(2) *Number and demarcation of defects*

single

double

diffuse: fine white lines or patchy

(3) *Location of defects*

gingival: one-half

incisal: one-half

occusal

cuspal

This index provides a descriptive classification based solely on clinical appearance. For aetiological studies, it provides the necessary information in detail because the recording system also allows for collecting information on medical and dental history. A number of studies using the FDI DDE Index have reported on its reproducibility. Clarkson & O'Mullane (1989) obtained a percentage reproducibility of 85% on a tooth surface basis, whereas Suckling & Pearce (1984) and Dummer et al (1986) reported values of 83% and 89%, respectively. The intra-operator reliability coefficient (Pearson's r) varied from 0.95 to 0.99 in the study of Clarkson & O'Mullane (1989). These reproducibility results show that, following calibration, examiners can produce consistent results using this index.

Modifications of the index have been proposed for different purposes (Fédération Dentaire Internationale 1992). These modifications allow for two simplified versions of the index, i.e. those suitable for screening surveys and general-purpose epidemiological surveys. Further variants of the index may be developed for other purposes. The advantage of this approach is that since the basic terminologies and criteria remain the same, whatever version of the index is used as most appropriate for the immediate purpose, results from different studies can still be compared, albeit with careful interpretation.

For studies in the future several points should be made clear: the version of the FDI DDE Index used; the derivation of the sample; the examination conditions, particularly lighting; the examination methods, particularly drying of the teeth; and examiner training and calibration.

Comparison of studies using the Fédération Dentaire Internationale Developmental Defects of Enamel Index

An example of how valid comparisons of studies highlight differences between population samples is shown in Table 1. These studies on samples of similar age in different countries were carried out by teams with at least one member involved in working groups involved in the development of the FDI DDE Indices (in 1982 and 1992). The results from England, Ireland and New Zealand are remarkably similar, whereas those from Hong Kong are much higher, suggesting real differences worth further investigation.

Exploring general factors in and between populations

An extension of this approach is where the same authors have examined different populations. Smith & Brook (1996a,b) have studied the prevalence of enamel defects

TABLE 1 Prevalence of defects in subjects aged 12–15 years using the Fédération Dentaire Internationale Developmental Defects of Enamel (FDI DDE) Index

<i>Per cent affected</i>	<i>Country</i>	<i>Reference</i>
95	Hong Kong	King & Brook (1984)
99	Hong Kong	King & Wei (1986)
63	New Zealand	Suckling & Pearce (1984)
65	New Zealand	Cutress et al (1985)
63	Ireland	Clarkson (1987)
68	England	Smith & Brook (1996a)

in a modern British population and a Romano-British sample from the third to fifth century AD using the same examiners, index and examination methods.

The modern sample consisted of 1518 white Caucasian school children aged 12–15 years living in East London. The overall prevalence of enamel defects in the permanent dentition of this group was 68.4%. Opacities (alterations in the translucency of the enamel) were present in 67.2% and hypoplasia in 14.6%. The per cent of children with both opacity and hypoplasia was 13.4%. The average number of teeth affected per individual with any enamel defect was 3.6, with 10.5% having 10 or more teeth affected. The teeth most commonly effected were the incisors and the premolars, and the surface most frequently involved was the buccal surface.

The Romano-British sample is from some five generations of a homogeneous population living in the third to fifth centuries AD, excavated from the Christian Cemetery at Poundbury, Dorset, UK. All 178 skulls with intact dentitions from the excavation were examined in a laboratory under good light. As the teeth were very dry, only hypoplastic defects were scored. Sixty-six skulls, i.e. 37%, had teeth showing hypoplastic defects, with a mean of five affected teeth per skull. The large majority of the defects were horizontal grooves, and in some instances there were several grooves on each tooth. The teeth most frequently affected were the canines followed by the incisors. The surface most often involved was the buccal surface. Only 1% of the defects occurred on the occlusal surface but this may be associated with the extensive attrition found in the sample.

A summary of the results is given in Table 2. The higher frequency of hypoplastic defects in Romano-Britons, their more widespread, often bilateral distribution, and their type and position suggests that they may be related to repeated illnesses between two and six years of age. The teeth and bones of the Poundbury sample have a high lead content (Waldron et al 1979, Stack & Whittaker 1982). Although this may not be a direct cause of enamel hypoplasia, it may have an indirect effect by increasing susceptibility to other diseases. In the modern sample the distribution of the few generalized hypoplastic defects was compatible with a major systemic disturbance

TABLE 2 Comparison of specific findings from studies of a modern and a Romano-British population

<i>Population</i>	<i>Prevalence of hypoplasia (%)</i>	<i>Percentage with four or more teeth affected</i>	<i>Prevalence of horizontal grooves (%)</i>	<i>Teeth most frequently affected</i>	<i>Surface involved</i>
Romano-British	37	25	30	Canines, incisors	Buccal (82%)
Modern	15	1	2	Incisors, premolars	Buccal (73%)

between six and 12 months of age. The absence of many similar defects in the Romano-Britons may be related to the high infant mortality.

Investigating factors in specific groups

Using the same standardized descriptive index it is possible to move from population studies to examining specific groups for indications of aetiological factors. As part of a longitudinal study of low birth weight children (i.e. those born weighing less than 2 kg), 110 children were examined at five years of age for the presence of enamel defects using the FDI DDE Index (Fédération Dentaire Internationale 1982). A control group of normal birthweight, healthy children matched for age, sex, race and social class was also examined. The clinical examination was conducted in a standardized manner, with the child upright in the chair. The teeth were cleaned with a toothbrush, dried with gauze and examined using a 60 w external light source and a illuminated mouth mirror. A photographic record was taken. Initial calibration of the examiner (J. M. F.) was undertaken against an examiner experienced in the use of the FDI DDE Index (J. M. S.). Ten per cent of the control children were examined by J. M. F. on two separate occasions, three months apart, with no access to previous records to test intra-examiner reproducibility. Using the method of Murray & Shaw (1979), the intra-examiner and inter-examiner reproducibilities were 74% and 67%, respectively.

Significantly more low birth weight children had enamel defects than controls (Fig. 1; low birth weight 77%, control 37% $\chi^2 = 32.7$, $p < 0.001$). This difference was significant only for hypoplasia, the hypoplasia category including those defects where there was both hypoplasia and opacity in the one lesion. Defects occurred most frequently on incisors (Fig. 2), the distribution of the defects sometimes, but not always, being symmetrical. If molars were also affected, the defects were often more marked on the first primary molar. In both low birth weight and control groups the defects occurred more often on the buccal than the lingual surface and more in the occlusal than the gingival half of the teeth.

The relationship of enamel defects in the low birth weight group to perinatal variables is shown in Table 3. There was no significant difference in the number of

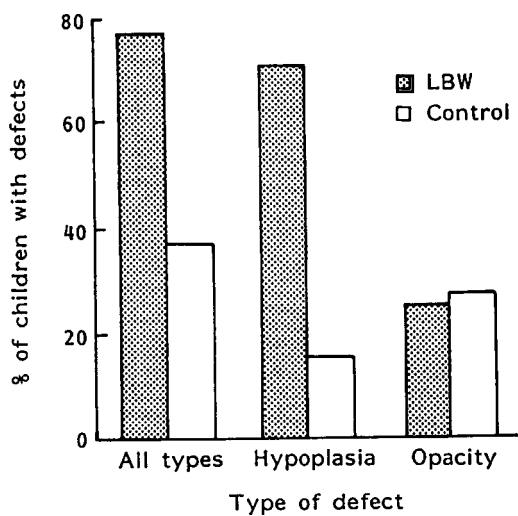


FIG. 1. The mouth prevalence of enamel defects in the primary dentition at age five years.

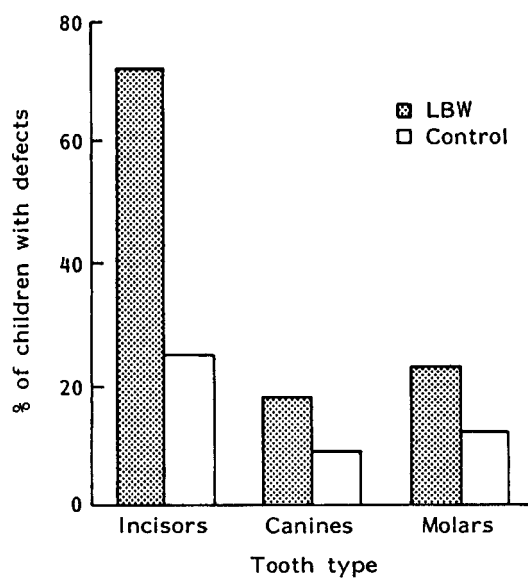


FIG. 2. The distribution of enamel defects in the primary dentition at age five years.

very low birth weight (≤ 1.5 kg) children with enamel defects compared with low birth weight (≤ 2 kg) children and no significant difference in defects between children born with birth weights appropriate for their gestational age compared with those small for gestational age. Children born at less than 32 weeks who had major or moderate neonatal illness, signs of birth asphyxia, or who required incubation on delivery, long-term ventilation or intravenous alimentation, more often had neonatal defects than children without these neonatal complications (Table 3). All nine children with cerebral palsy had enamel defects, but they also had one or more of the above neonatal complications significantly associated with an increased frequency of enamel defects.

Log linear modelling, a multivariate method, was used to determine how specific perinatal variables were associated with defect formation. The association between ventilation and the presence of defects was strong ($p < 0.001$).

The higher frequency of enamel defects in low birth weight children compared to controls is in agreement with Grahnén et al (1974), Johnsen et al (1984) and Soew et al (1987). The overall higher prevalence figures for both low birth weight and control groups in our study compared to these three studies almost certainly reflects the sensitivity of the FDI DDE Index and also the need to examine all surfaces of all teeth. Interestingly, the prevalence figures for the controls in our study relate closely to those of Murray & Shaw (1979) for the primary dentition in a population study using a descriptive index which was a precursor of the FDI DDE Index.

Disturbances of vitamin D metabolism and mineral substrate deficiency are well recognized in low birth weight children (MacMahon et al 1989). Mineral substrate deficiency may underlie many of the enamel defects seen in low birth weight children. It is possible that low blood oxygen may be associated with the enamel defects seen in our study in infants requiring more than four hours of ventilation. Johnsen et al (1984) suggested that the increased prevalence of enamel defects in children with respiratory distress syndrome was a function of ameloblast sensitivity to oxygen deprivation. Asymmetrical defects of incisors were seen in our study in several children who required oro-endotracheal tubes. Moylan et al (1980) and Soew et al (1984) have related enamel defects of incisors in low birth weight children to traumatic forces exerted during laryngoscopy and oral intubation.

It is probable that the defects result from the cumulative severity, duration, interactions and timing of insults as well as their nature. This contention is supported by the work of Engstrom & Noren (1986) who applied orthodontic forces to developing rat incisors and produced an increased prevalence of disturbances of enamel formation in those animals who were hypocalcaemic compared to normocalcaemic animals.

Conclusions

Enamel defects are present as a range of clinical features for which a variety of terminology and indices for measurement have been used, rendering valid comparisons between different studies difficult.

TABLE 3 Prevalence of enamel defects in the primary dentition of low birth weight children related to perinatal variables at age five years

<i>Perinatal variables</i>	<i>Total</i>	<i>Enamel defects</i>	<i>Significance (χ^2)</i>	<i>95% confidence interval for difference</i>
Birth weight				
<1.5 kg	60	50 (83%)	$p=0.15$	- 2.5% to 29%
> 1.5 kg	50	35 (70%)		
Gestation				
< 32 weeks	65	55 (85%)	$p=0.05$	1.65% to 43%
> 32 weeks	45	30 (67%)		
Intra-uterine growth				
AGA	61	48 (79%)	$p=0.8$	- 12% to 20%
SGA	48	36 (75%)		
Neonatal problems				
major	24	23 (96%)	$p=0.02$ (Kendall's tau)	Minor to moderate 15% to 46%, minor to major 18% to 46%
moderate	19	18 (95%)		
minor	64	41 (64%)		
Birth asphyxia (Apgar scores)				
1 m < 3	32	29 (91%)	$p=0.04$	6% to 36%
1 m > 3	73	51 (70%)		
Incubation on delivery				
Yes	51	45 (88%)	$p=0.01$	7% to 38%
No	55	36 (65%)		
Phototherapy				
Yes	53	45 (85%)	$p=0.06$	1.3% to 34%
No	49	(33 (67%))		
Long-term ventilator				
Yes	29	29 (100%)	$p=0.001$	22% to 42%
No	78	53 (68%)		
Intravenous alimentation				
Yes	31	29 (94%)	$p=0.02$	10% to 37%
No	76	53 (70%)		

AGA, appropriate for gestational age; SGA, small for gestational age.

There is now a standard descriptive index with clear criteria available which has been shown to have good reproducibility and is available in several forms for different types of study (Fédération Dentaire Internationale 1982, 1992).

From studies of different populations in several countries using the FDI DDE Index by calibrated examiners it has been possible to suggest similarities and real differences worth further study in exploring the aetiology of enamel defects.

By comparing an ancient and a modern British population using the same examiners and index, it is suggested that not only may episodes of systemic illness cause enamel defects but also that general debilitating factors, in this instance ingestion of high levels of lead, may have an indirect effect by increasing susceptibility to disease.

From a detailed study of a specific group, i.e. low birth weight children, in comparison to a control group, again using the FDI DDE Index, evidence has been advanced for the role of some specific factors — deficiencies of vitamin D metabolism and mineral substrate, oxygen deprivation and trauma from endotracheal tubes — in contributing to the development of enamel defects. However, it is probable that enamel defects result from the cumulative severity, duration, interaction and timing of insults as well as their nature and the individual susceptibility.

The remit of this chapter was to address the role of environmental factors, and it has been shown that not only may there be specific factors (systemic and local) but also that there are background factors influencing the individual's response. In considering the full picture of the aetiology of enamel defects, genetic as well as environmental factors must be considered. This includes not only those genetic factors in specific conditions considered elsewhere in this volume but also those involved in tooth development and the individual's resistance to disease.

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DISCUSSION

Snead: Have you looked at human populations that are experiencing macrobiotic nutritional deficiencies to see if there is any correlation between these dietary changes and the prevalence of specific enamel defects? One of the charity organizations has monitored nutritional status in South American children, and compared those who

have received dietary supplements with those who have not. This might be a good group for you to study.

Brook: This is a super idea. We have not done it yet but it's a logical extension of our current studies. For example, some of the differences between the Romano-British group and the modern Britons may well be due to nutrition and illness. Although we are dealing with a multifactorial condition, as long as we obtain solid data, for example in the low birth weight children, we can identify the important factors and see if they correlate with the laboratory findings.

Winter: There have been studies in the developing world. For example, in Guatemala Sweeney et al (1969) found linear enamel hypoplasia, which they attributed to low levels of vitamin A in cord blood serum at birth combined with early infantile illness. However, the problem with those data and also the data from French Polynesia was the confusion between linear enamel hypoplasia and early or arrested enamel caries (Baume 1969). In both these studies linear enamel hypoplasia was said to be limited to the maxillary primary incisors, whereas neonatal enamel hypoplasia affects primary incisors, canines and first molars in both upper and lower jaws. Early or arrested rampant carious lesions of the primary maxillary anterior teeth may resemble enamel hypoplasia and cause confusion in epidemiological studies.

Brook: I agree that much of the existing data are not suitable for comparisons to be made because of different definitions of hypoplasia. It is important that a standard definition is used and that we can also rely on the nutritional measurements.

Thesleff: I wonder whether we would observe similar defects in developed countries. We have seen in Finland during the last few years many cases of dramatic enamel hypoplasia of the first molars in children who have very little hypoplasia in other teeth. Satu Alaluusua at our Institute of Dentistry found this defect was correlated with the concentration of dioxins in mothers' milk (Alaluusua et al 1996).

Brook: There is definitely a problem in identifying the cause of enamel defects that occur on first permanent molars and not on incisors. It is possible that for some reason certain genetic defects can just affect particular teeth. However, Joyce Smith studied a group of 18 such index cases and their families, and her data were inconclusive as to specific genetic factors or illnesses (Smith 1984). It's interesting that people are looking at possible environmental influences but the problem of how they affect only certain teeth still remains.

Alfred: At what point do we go from saying there's developmental defect in enamel to saying that it is amelogenesis imperfecta. If there's a clear inheritance pattern then we say it's amelogenesis imperfecta, but when we are simply presuming that it is inherited where do we draw the line? Also, I am unhappy with the use of the terms hypocalcification and hypomaturation and I prefer to use the term hypomineralization. My colleague, Peter Crawford, refers to enamel defects in the first permanent molars as dysmineralization because the abnormally mineralized enamel could be caused by a range of effects. It is possible that this sort of approach (using novel terms) could solve some of the terminology problems.

Wright: In all the known cases of 'dysmineralization' in humans the defects are actually hypomineralized, and there aren't any that are hypermineralized. Therefore, hypomineralized is an appropriate term and it is unnecessary to confuse the terminology further.

Aldred: Alan Brook also mentioned that 1% of the Romano-British population had vertical grooving of the teeth. Could this represent the earliest example of X-linked amelogenesis imperfecta?

Brook: That's an interesting thought. However, again the problem is being able to determine whether the defect is hereditary. We have looked in this population at the inheritance of hypodontia by plotting those affected against the site of excavation. We found that there is some degree of clustering, suggesting that these defects may well be occurring in families. We could also do a similar plot for the vertical grooving because the evidence points to family burials at these excavation sites.

Aldred: There was one report in the literature of a native American Indian population in which there was a skull that had vertical grooving (Cook 1980). I looked at the records of that excavation site to see if there was any evidence that it was a female skull and, if so, whether there might be a corresponding male with uniformly affected enamel. However, there was no evidence that this was the case.

Hammarström: In children born in the early 1970s in Sweden there was a dramatic increase in the number of children with a special type of mineralization disturbance of the first permanent molars. In some children only one tooth was affected, in others two, three or all four first molars were affected. Clinically, the occlusal surfaces were brownish, soft and hypocalcified. The teeth were also sensitive to hot food. In addition, the maxillary medial incisors were sometimes affected, but to a lesser degree. We had the opportunity to study the mineralization pattern in some of these teeth by means of microradiography. In about 50% of the cases there were diffuse mineralization disturbances in the whole enamel, whereas in the other 50% the cervical third of the enamel appeared normal. The studies were never published because we did not know the cause of the disturbances.

Slavkin: I have a different interest in the implications of Alan Brook's work. I am studying craniofacial birth defects, and, in particular, the cleft lip and cleft palate. It's clear that the clefted phenotype means very little with respect to understanding the mechanism that has resulted in an individual defect because the defect can result from a broad range of environmental 'insults' and, therefore, we now assume that there are many different genes that regulate pathways which produce cleft lip and/or palate. Therefore, I am concerned about the pragmatic applications of how we can improve health promotion, diagnosis and therapeutics on one hand, and how we can improve our understanding of the basic mechanisms on the other. One area that Alan Brook did not mention was the field of molecular epidemiology. Perhaps high through-put genotyping would be useful to define the genetic risk factors that can produce the amelogenesis imperfecta phenotype.

Brook: It's a valuable area and one that I would like to investigate, but the problem is where to start looking if we haven't yet defined the group that we want to examine.

Another way forward—which we have used to study variations in tooth size and number, and may also be used to study the cleft lip and cleft palate—is to develop a statistical model that initially incorporates all the factors so that it sets up a series of predictions that you can then investigate in the families.

Jones: In the Romano-British population you were limited to studying only 178 cases out of a possible 1000 because you chose to analyse intact tissues. This is a small proportion, so in terms of doing a population study it is severely flawed.

Brook: We had to accept that problem. If we had included fragments of tissues in our study we would have biased the data in a different way; for example, more of the incisors may have been retained by chance.

Snead: In the Romano-British populations one might not expect there to have been a decrease in the incidence of *amelogenesis imperfecta* because of the Eve effect, i.e. the genetic bottleneck acting upon the X chromosome. Over 40% of the children in the age-matched control group had marked enamel defects, suggesting that teeth are sensitive to many factors and, as a result, make a poor reporter. Why don't we see a higher prevalence of enamel defects in wild animals, such as in the rhinoceros that Alan Boyde has studied? It's remarkable that human teeth seem to be so much more sensitive than other animals.

Boyde: In X-linked *amelogenesis imperfecta* vertical grooving is observed because there are two populations of ameloblasts: those that make a small amount of enamel and those that make more. In the rhinoceros there are also two populations of ameloblasts: those that move down the tooth and those that move up. This also results in vertical grooving (Boyde & Fortelius 1986). Therefore, vertical grooving of the enamel occurs in both cases, but for completely different reasons.

Wright: Has anybody looked for similar enamel defects in animal models that have a similar duration of dental development, such as in primates?

Aldred: I'm not aware of any data on this, and we have looked extensively at the literature. Rushton (1964) suggested that *Musschenbroek's* palm civet was an animal model for X-linked *amelogenesis imperfecta*. We went to the British Museum and examined the remains of the body and found that it was a male, so it could not have been an example of lyonization.

Robinson: I agree that although the overall chemistry of enamel is similar in different species, the developmental time courses, and particularly those of the maturation stage, are very different. The width of the maturation zone also varies; for example, a 2 mm zone of porosity moves down a cow tooth as it matures but in a deciduous human tooth it occupies almost the whole surface. There is an additional complication in that, at least in cows, at different points along the surface the timing of maturation differs. Therefore, the timing of the most sensitive period in development changes along the tooth.

Boyde: It would be interesting to recover some more biological information from these patients about the nature of the event leading to hypoplasia and what actually happens with the ameloblasts. This information could be obtained by scanning electron microscopy. One would expect that in hypoplasia, the ameloblasts might

lose their function in the middle of the secretory phase of amelogenesis. In other words, there would be active secretory phase pits and all the normal signs of decussation. We have looked at hypoplastic teeth and shown that this is true. In a moderate case of hypoplasia, the ameloblasts might recover and make a modest attempt at making an enamel surface but with shallower pits. One could therefore determine whether oxygen deprivation at birth could lead to the total cessation of secretory activity, but with the ability to recover and enter the maturation stage. The advantage of this type of analysis would be that it would not necessarily require tooth extraction. One could simply make a silicone rubber impression of the tooth as soon as it erupted. This may be more valuable than waiting for a few years to study the defects.

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Determinants and mechanisms of enamel fluorosis

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Abstract. Enamel fluorosis occurs when fluoride concentrations in or in the vicinity of the forming enamel are excessive during its pre-eruptive development. Fluoride concentrations in plasma, enamel and other tissues reflect the difference between intake and excretion, i.e. fluoride balance. In addition to the diet, modern sources of ingested fluoride include a variety of dental products, some of which have been identified as risk factors for fluorosis. Fluoride absorption is inversely related to dietary calcium which, at high concentrations, may cause net fluoride secretion into the gastrointestinal tract. The excretion of absorbed fluoride occurs almost exclusively via the kidneys, a process which is directly related to urinary pH. Thus, fluoride balance and tissue concentrations and the risk of fluorosis are increased by factors such as high protein diets, residence at high altitude, and certain metabolic and respiratory disorders that decrease pH. Factors that increase urinary pH and decrease the balance of fluoride include vegetarian diets, certain drugs and some other medical conditions. Although several other fluoride-induced effects might be involved in the aetiology of fluorosis, it now appears that inhibition of enzymatic degradation of amelogenins, which may delay their removal from the developing enamel and impair crystal growth, may be of critical importance. In addition to the effects of fluoride, disturbances in enamel formation that can be confused with fluorosis are caused by chronic acidosis and hypoxia independently of the level of fluoride exposure.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 226-245

It is generally accepted that dental fluorosis results from elevated fluoride concentrations in, or in the vicinity of, the developing enamel. As it has not yet been possible to directly measure such concentrations in the several anatomically and functionally distinct subcompartments of developing enamel, research attention has focused on concentrations in plasma, the fluid which delivers fluoride to enamel and all other tissues. Plasma concentrations are mainly determined by two factors: intake (Guy 1979) and the metabolic processes responsible for the retention or the elimination of fluoride (Whitford 1996).

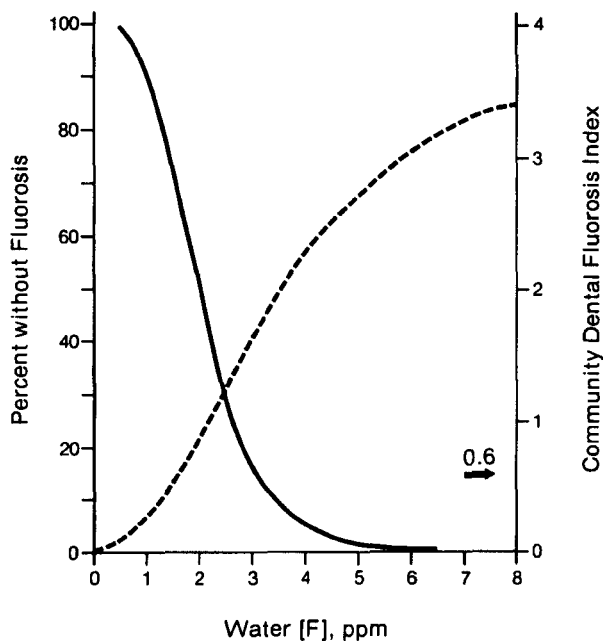


FIG. 1. The relationship between drinking water fluoride concentrations and the prevalence of dental fluorosis (solid line) and the community dental fluorosis index (dashed line) as determined in the USA in the 1930s to 1940s, i.e. before fluoride-containing dental products were available. An index value of 0.6 was considered by Dean (1942) to be the threshold for a public health problem in a community. It occurred when the water concentration was 1.6–1.8 ppm.

Intake

The epidemiological investigations conducted in the USA about 60 years ago defined the relationship between drinking water fluoride concentrations and the prevalence and severity of dental fluorosis (Fig. 1 and Dean 1942). Compared with those findings, recent epidemiological studies have documented a marked increase in the prevalence of dental fluorosis (Szpunar & Burt 1987, Pendrys & Stamm 1990). The greatest increase has occurred in communities without fluoridated water. Fluorosis in the USA is now more than twice as prevalent in optimally fluoridated communities but more than 10 times as prevalent in communities with low water fluoride concentrations. This indicates that fluoride intake and retention by children at risk have increased significantly.

Dietary fluoride intake has remained relatively constant since the 1940s (Burt 1992). This and the increased prevalence of dental fluorosis prompted investigators to seek the sources of additional fluoride intake. Leverett (1982) was among the first to point out that inadvertent or intentional fluoride ingestion associated with the use of dental

products by children could be sufficient to cause fluorosis. Subsequently, several investigators have concluded that dietary fluoride supplements and the early use of a fluoridated dentifrice are risk factors (Osuji et al 1988, Pendrys & Katz 1989). Mouthwashes, although used by fewer children, also contribute to the risk. The combined use of these products significantly increases the risk that dental fluorosis of some degree will occur (Fig. 2).

Studies of the amounts of toothpaste used by children have indicated that the range is 0.1–2.0 g per brushing, with an average of about 1.0 g per brushing (Hargreaves et al 1972, Dowell 1981, Brunn & Thylstrup 1988). For a 1000 ppm dentifrice, therefore, the average amount of fluoride introduced into the mouth with each brushing is 1.0 mg, an amount similar to that used with an over-the-counter mouthwash. The fractions of fluoride introduced into the mouth with these products that are ingested range from about 10% to nearly 100%; the average being about 25% or 0.25 mg. The

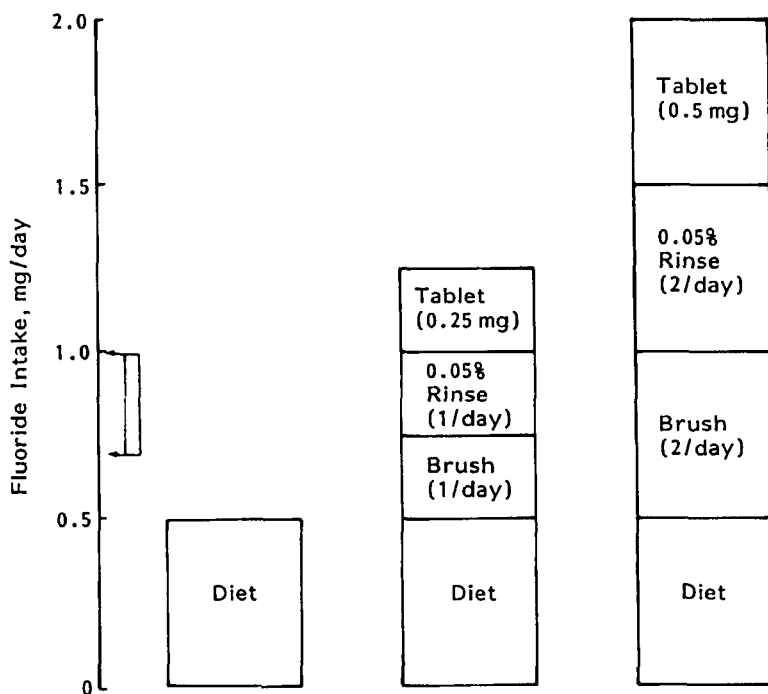


FIG. 2. The estimated average total daily fluoride intake with the diet and dental products by children whose drinking water contains fluoride at about 1.0 ppm. In the absence of tooth brushing, mouth rinsing or the use of fluoride tablets, the average intake by diet is 0.5 mg/day. The range of intake (0.7–1.0 mg/day) expected to produce a community fluorosis index value of 0.6 is shown on the y-axis.

amount ingested is higher among younger children, due in part to inadequate control of the swallowing reflex.

Figure 2 shows the additive effects of using some of the fluoride-containing products on the daily intake of fluoride by young children whose drinking water is optimally fluoridated. The range of intake that would be expected to produce some degree of dental fluorosis is indicated by the arrows on the y-axis (Whitford et al 1987). If the diet were the only source of fluoride, then intake would be about 0.5 mg/day (Burt 1992). Using the average retention value of 25%, brushing the teeth only once a day increases the intake close to the threshold for some degree of fluorosis. Brushing more frequently and/or using other products produces a similar or an even greater risk.

As noted above, the data in Fig. 2 are based on averages. At any age, however, there is a wide range of fluoride intake among individuals. This explains, at least in part, both why some individuals living in the same community and with similar dietary fluoride intakes have dental fluorosis while others do not, and the degrees of severity among affected individuals. The quantitative aspects of fluoride metabolism, however, can also span a wide range and contribute to the body burden of fluoride and the expression of dental fluorosis.

Dose schedule and plasma concentrations

Based on the study with rats conducted by Angmar-Månsson et al (1976), it was thought that dental fluorosis was the result of occasional spikes in plasma fluoride concentrations that reached values of about 10 $\mu\text{mol/L}$. That such spikes could produce fluorosis was confirmed by a study in which rats received daily fluoride injections for one week (Angmar-Månsson & Whitford 1982). It was also found that once- or twice-daily injections that caused peak concentrations of 5 $\mu\text{mol/L}$ did not produce fluorosis. This suggested that the total daily dose was not the important variable but instead it was the amount of fluoride given at one time and the resulting peak plasma fluoride concentrations.

The latter study, however, also included two groups that received fluoride during the week by continuous infusion from miniosmotic pumps implanted subcutaneously. This method of administration produced slightly elevated but relatively constant plasma concentrations that averaged 3.3 and 4.7 $\mu\text{mol/L}$ during the week. Dental fluorosis was present in some of the 3.3 $\mu\text{mol/L}$ rats and all of the 4.7 $\mu\text{mol/L}$ rats. It was concluded, therefore, that high peak plasma fluoride concentrations were not necessary to produce disturbances in enamel mineralization. This was confirmed by the results of a subsequent study with rats that were infused with fluoride for eight weeks (Angmar-Månsson & Whitford 1984). Fluorosis was evident in all rats of both groups even though their average plasma fluoride concentrations were only 1.5 and 3.1 $\mu\text{mol/L}$. Concentrations within this range would be expected in humans whose drinking water contains fluoride between about 2 and 4 ppm and whose enamel also exhibits some degree of fluorosis.

Based on these results, it can be concluded that the risk of dental fluorosis is directly related to the interaction of circulating fluoride concentrations and time, i.e. the area under the time-concentration curve. Thus, it appears that dental fluorosis can result from a range of plasma fluoride concentrations provided that they are maintained for sufficiently long periods.

Despite this, however, it is possible to produce fluoride concentrations in the vicinity of the developing enamel, but not in plasma, sufficient to cause fluorosis (Angmar-Månsson & Whitford 1985). This study, which used the continuously erupting rat incisor as the model, was based on the hypothesis that the long-term mobilization of fluoride from bone following a single dose could increase enamel concentrations in the absence of elevated systemic concentrations. Six groups of rats were given single doses of fluoride ranging from 0 to 14 mg/kg by intraperitoneal injection. Plasma concentrations returned to baseline values within 24–48 h. The mandibular incisors were examined grossly and microradiographically at 15, 35 and 70 days after the injections. At 15 days, disturbances in enamel mineralization were seen in all fluoride-dosed groups. At 35 days, fluorosis was evident in the three groups that received 4 mg/kg or more. After 70 days, the incisors of the 14 mg/kg group still showed evidence of fluorosis.

The incisors from rats sacrificed at 35 and 70 days (by which time they would have been renewed nearly twice) were subsequently analysed for fluoride by nuclear microprobe (Angmar-Månsson et al 1990). They were found to have elevated concentrations that were proportional to the doses given weeks earlier (Fig. 3). The findings strongly support the hypothesis that, following the pulse loading of bone, fluoride is slowly released by exchange and/or remodelling from bone supporting the developing tooth. Thus, locally elevated fluoride concentrations sufficient to affect amelogenesis adversely can be produced in the vicinal fluids of the developing enamel in the absence of elevated systemic concentrations. It is not certain whether fluoride mobilized from bone surrounding developing teeth or from resorbing bone during tooth eruption normally contributes to human fluorosis but these findings support that possibility.

Absorption

Under most conditions the absorption of ingested fluoride is rapid ($t_{1/2}$ c. 30 min) and 80–90% complete. Plasma fluoride concentrations begin to increase during the first few minutes after ingestion, reach their peak after 30 min and then decline exponentially to preingestion concentrations during the next few hours, depending on the size of the dose.

pH-dependent absorption by non-ionic diffusion begins in the acidic environment of the stomach where ionic fluoride is converted to the undissociated weak acid, HF ($pK_a = 3.4$), the form in which fluoride appears to cross most cell membranes and epithelia (Whitford & Pashley 1984, Whitford 1996). Absorption from the stomach can account for up to 40% of the ingested amount, depending on pH and the rate of

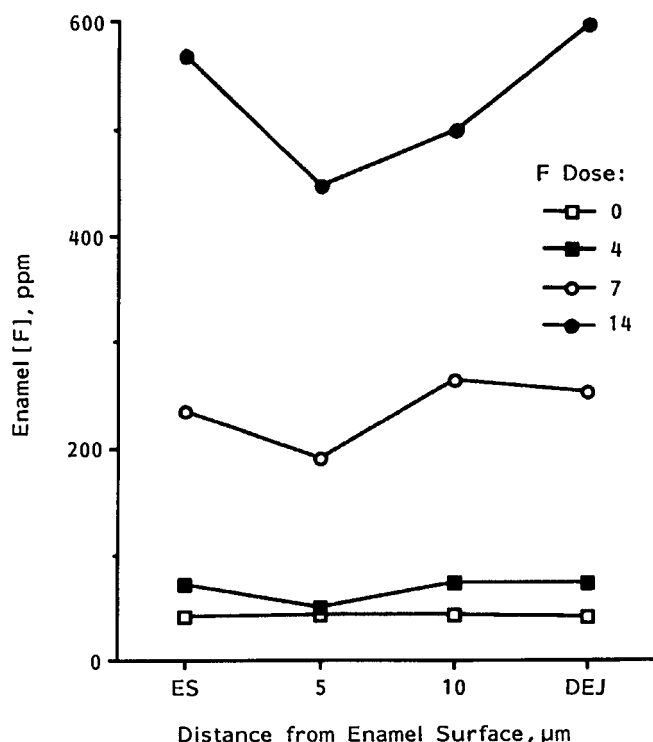


FIG. 3. Fluoride concentrations in developing enamel of rat mandibular incisors as a function of distance from the enamel surface. Fluoride had been administered as a single intraperitoneal injection 70 days earlier. Doses in mg/kg.

gastric emptying. The administration of fluoride in an acidic solution or during active gastric acid secretion results in earlier and higher peak plasma concentrations. The ultimate extent of absorption, however, is not much affected by gastric acidity due to further absorption from the small intestine by an unidentified mechanism that is not pH dependent (Messer & Ophaug 1993). Faecal fluoride is generally regarded as unabsorbed but results published recently suggest that this is not true under all conditions (Whitford 1994).

In view of the extensive absorption that typically occurs, it is clear that the bioavailability of fluoride can be decreased more than it can be increased. Certain di- and trivalent cations react with fluoride to form insoluble and poorly absorbed compounds. Calcium has been studied most, although magnesium, iron and aluminium also have this effect. Budden et al (1988) concluded that the response by osteoporotic patients to fluoride therapy depends to a significant degree on the extent of absorption and, hence, the availability of the ion to bone. The average

plasma fluoride concentrations of their patients increased by 54% when supplemental calcium and vitamin D were excluded from the therapeutic regimen.

In addition to reducing fluoride absorption, an increased dietary calcium concentration (1.4% by weight) has been shown to promote net fluoride secretion into the gastrointestinal tract of rats (Whitford 1994). In this study, plasma fluoride concentrations were elevated by constant infusion using miniosmotic pumps implanted subcutaneously. Faecal fluoride excretion exceeded dietary fluoride intake by as much as 150%, which indicated a substantial net migration of systemic fluoride into the gastrointestinal tract. Compared with the group fed the control diet (0.4% Ca^{2+}), this was associated with lower plasma and bone fluoride concentrations and lower urinary fluoride excretions. Conversely, Beary (1969) and Riggins et al (1974) found that rats fed a diet low in calcium had higher bone fluoride concentrations than those fed a diet adequate in calcium, an effect consistent with increased absorption.

Thus, it is clear that gastric pH influences the rate of fluoride absorption and the peak concentrations of the ion in the body fluids. Further, the dietary concentration of calcium can influence the extent of absorption and thus affect fluoride concentrations throughout the body. Although not yet studied directly, there is adequate justification for the prediction that these variables affect the expression of dental fluorosis in humans.

Rate of mineralization

The fate of absorbed fluoride is almost entirely accounted for by calcified tissue uptake and urinary excretion. While there is considerable variation among individuals, about 50% of the fluoride absorbed by healthy, young or middle-aged adults each day is excreted and the remainder is acquired by calcified tissues. This distribution is shifted strongly in favour of retention during growth in young children (Ekstrand et al 1994a) and young dogs (Fig. 4 and Whitford 1996) due to the large surface area of the numerous and loosely organized crystallites in the developing skeleton. The rate of fluoride uptake by enamel is also greatest during rapid mineralization in the late secretory and early maturation phases, probably for the same reason. Weatherell et al (1977) reviewed this subject and concluded that the fluoride concentrations in the developing enamel of several species, especially around the transitional zone, are relatively high.

More recently, epidemiological studies of human populations have indicated that developing enamel is most susceptible to fluorosis in the late secretory or early maturation phase (Pendrys & Katz 1989, Evans 1989). This is consistent with the observation of high fluoride concentrations in transitional enamel. These findings suggest that the permanent anterior teeth, which are of the most important aesthetic concern, are at greatest risk for fluorosis during a two-year period extending through the second and third years of postnatal life.

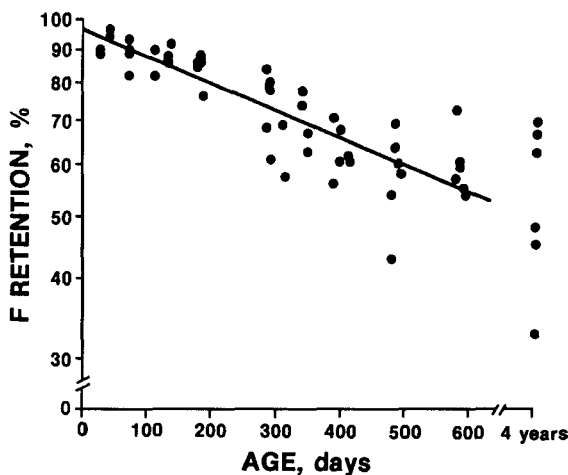


FIG. 4. The retention of fluoride as a function of age by growing dogs in a longitudinal study. At the time of weaning, about 90% of the fluoride dose was retained; in adulthood 50% was retained. Thus, urinary excretion accounted for only 10% of the dose at the youngest age, whereas it accounted for 50% in the adults. Similar values occur in humans.

Renal excretion

The kidneys are the major route for the elimination of fluoride from the body. Compared with the renal clearances of other halogens (typically $<1\text{--}2\text{ mL/min}$), the clearance of fluoride is high, although there is considerable variation among individuals. For example, Schiff & Binswanger (1982) reported clearances ranging from 12 to 71 mL/min among their subjects. Ekstrand et al (1994b) determined renal clearances in infants 37–410 days old. The average value was 6.3 mL/min but individual values ranged from 1.9 to 12.1 mL/min.

The renal clearance of fluoride (C_F) is directly related to glomerular filtration rate (GFR) and urinary pH. When GFR in adults declines to about one-third of normal on a chronic basis, the reduced excretion leads to measurable increases in the fluoride concentrations of plasma and other tissues (Waterhouse et al 1980). This is probably true for children as well, although no data are available to support this possibility.

The importance of urinary pH to the renal clearance of fluoride was discovered in studies with rats (Whitford et al 1976) and has been confirmed in other species including humans. The pH dependence of the clearance, like that for gastric absorption, is consistent with the hypothesis that the undissociated acid, HF, is the form in which fluoride permeates the tubular epithelium. The fraction of fluoride in the tubular fluid that exists as HF increases as the pH becomes more acidic. This increases the transepithelial HF concentration gradient, which promotes tubular reabsorption and thus reduces the clearance. In a more alkaline tubular fluid, the

fraction of fluoride that exists in the poorly permeating ionic form increases, which reduces reabsorption and increases the clearance. Since renal excretion accounts for a substantial portion of the fate of absorbed fluoride, changes in urinary pH can affect the retention, tissue concentrations and effects of the ion.

Factors that affect urinary pH include: acidifying (ammonium chloride, several diuretics) or alkalinizing (acetazolamide, sodium bicarbonate) drugs; certain metabolic and respiratory disorders such as diabetes mellitus, renal tubular acidosis and chronic obstructive pulmonary disease; the composition of the diet; and the hypoxia associated with residence at high altitude.

Figure 5 shows the enamel fluoride and phosphorus concentrations of three groups of rats that differed in acid-base status for 30 days (Whitford & Reynolds 1979). The acidotic group received drinking water containing NH_4Cl , the alkalotic group

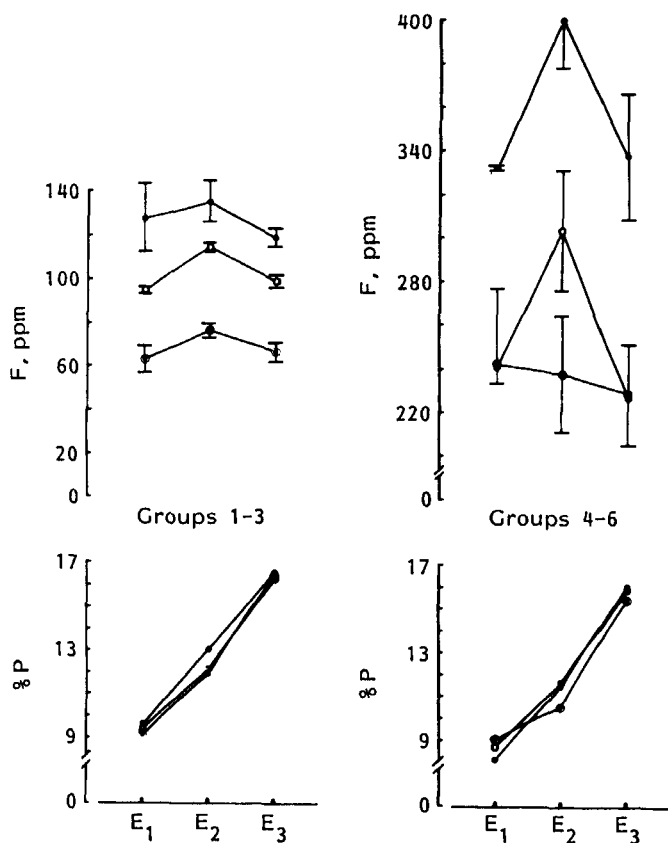


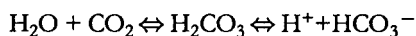
FIG. 5. Fluoride and phosphorus concentrations of developing enamel from rat mandibular incisors. Groups 1-3 received distilled water while Groups 4-6 received water containing 50 ppm fluoride for 30 days. Acid-base status: acidosis (●); normal (○); alkalosis (◐). Enamel sections: E₁, secretory; E₂, transitional; E₃, maturing.

received water containing NaHCO_3 and the control group received distilled water containing no additives. Fluoride (50 ppm) was added to the water of one-half of the rats in each group. At the end of the study the mandibular incisors were removed and three sections of developing enamel were collected separately from each rat. There were no significant differences in phosphorus concentrations attributable to acid-base status. The enamel fluoride concentrations in the acidotic group, however, were significantly higher than those of the alkalotic group and, in several cases, higher than those of the control group. Plasma fluoride concentrations showed the same pattern. Further, although incisor enamel fluorosis was present in each of the groups given fluoridated water, it was most apparent in the acidotic group.

It is noteworthy that chronic acidosis *per se* causes disturbances in enamel mineralization in both rats and dogs without exposure to fluoride (Angmar-Månsson & Whitford 1990, Whitford & Angmar-Månsson 1995a,b). The mechanism for this effect, which can be extremely severe, remains unknown but may be related to the formation of a higher than normal proportion of the relatively soluble dicalcium phosphate dihydrate (brushite) that tends to form in acidic environments.

Several reports have described the effects of acid-base changes induced by drugs or diet on the pharmacokinetics of fluoride in human adults. In a study by Ekstrand et al (1980), five healthy subjects ingested NH_4Cl to acidify the urine (pH 5.25) and then NaHCO_3 to alkalinize the urine (pH 7.43). The average renal clearance was 62 mL/min when the urine was acidic and 98 mL/min when it was alkaline. The plasma half-lives were also significantly different (4.3 vs. 2.4 h). Similar results were reported by Whitford & Weathered (1996), in which six subjects participated in a double crossover study. The subjects consumed a meat and dairy product diet to acidify the urine (pH 5.59) and then a vegetarian diet to alkalinize the urine (pH 7.22). The average renal clearances were 0.89 mL/min per kg body weight when the urine was acidic and 1.08 mL/min per kg body weight when it was alkaline. In acidic and alkaline urine the fractional clearances (C_F/GFR , i.e. the percentage of fluoride entering the renal tubules by glomerular filtration that are actually excreted) were 53% and 72%, respectively, and the plasma half-lives were 3.9 and 3.2 h, respectively. These results strongly suggest that diet-induced changes in urinary pH can affect the renal handling of fluoride such that long-term differences in the retention and tissue concentrations could decrease (alkaline urine) or increase (acidic urine) the risk of dental fluorosis.

Residence at high altitude is associated with a chronic, incompletely compensated respiratory alkalosis caused by hypoxic stimulation of the respiratory centre. The increased rate of breathing raises the O_2 content of the blood but reduces the CO_2 content. Consequently, the following reaction is forced to the left, which lowers the hydrogen ion concentration in the blood.



As shown in Fig. 6, urinary pH increases transiently during the initial development of the alkalosis, an effect due mainly to the low blood PCO_2 that slows the renal

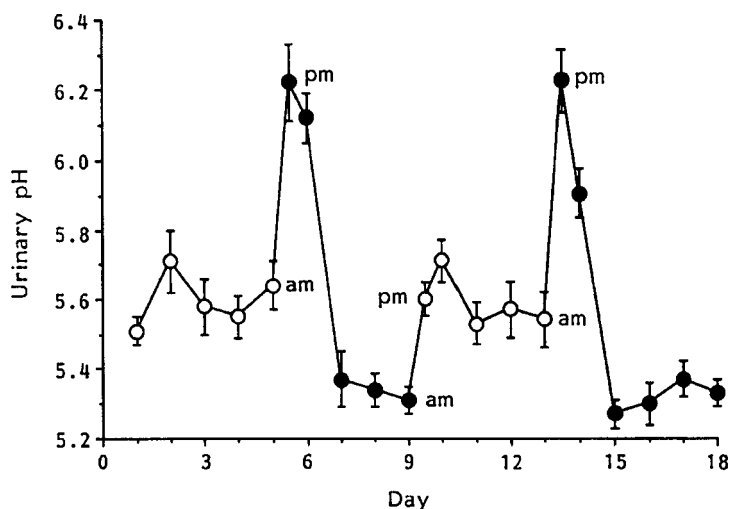


FIG. 6. The effect of hypobaric hypoxia (simulated high altitude) on urinary pH in rats. On Days 1–4 the animals were at sea level (○). After the collection of urine on the morning of the fifth day, the rats were exposed to a simulated altitude of 18 000 ft (●) until the afternoon of the ninth day when they were returned to sea level. Note the transient increase in pH followed by a sustained decrease on Days 7–9. The same effects occurred when the sequence was repeated.

reabsorption of bicarbonate. After several hours, however, urinary pH begins to fall as the filtered load of bicarbonate declines. A relatively acidic urine is then excreted as long as the hypoxic stimulation of respiration persists. It was hypothesized, therefore, that long-term residence under hypoxic conditions would result in reduced urinary fluoride excretion and increased tissue fluoride concentrations. This has been tested in several experiments, the most recent of which will be described here (Whitford & Angmar-Månsson 1995).

One group of rats was raised at sea level and another in an environmental chamber adjusted to a simulated altitude of 18 000 feet for five weeks. Two subgroups were formed in each of the main groups based on the fluoride concentrations of the drinking water (0 and 25 ppm). Forty-eight-hour food and water intake determinations and urine and faeces collections were made twice each week. These samples were analysed so that fluoride absorption, excretion and balance could be determined. At the end of the study, the transitional enamel of the mandibular incisors was analysed for fluoride.

Table 1 shows the intake, excretion, absorption, balance and enamel fluoride concentration data. Intake did not differ between the two low fluoride groups nor the two groups given water with 25 ppm fluoride. Urinary fluoride excretion was slightly lower in the two hypoxic groups, which was consistent with the lower average urinary pH values of these groups (5.3 vs. 5.8). Faecal fluoride excretion was

TABLE 1 Fluoride intake, excretion, absorption and balance data, and incisor transitional enamel fluoride concentrations of rats as functions of fluoride exposure and altitude of residence for five weeks

Fluoride exposure/ altitude ^b	Intake ^a		Excretion ^a				Total	Absorption	Absorption %	Balance	Retention %	Enamel [F] ppm
	Food	Water	Total	Urine	Faeces	Total						
Sea level	0.064, 0.002	0	0.064, 0.002	0.019, 0.003	0.013, 0.001 (a)	0.032, 0.003	0.051, 0.002	79.53, 1.39 (a)	0.032, 0.002	50.52, 3.77 (a)	7.58, 0.29 (a)	
18 000 ft	0.064, 0.003	0	0.064, 0.003	0.015, 0.002	0.010, 0.001 (b)	0.025, 0.002	0.054, 0.003	83.89, 1.47 (b)	0.039, 0.004	59.45, 3.58 (b)	15.06, 1.35 (b)	
Sea level+F	0.068, 0.004	2.529, 0.050	2.597, 0.052	0.129, 0.022	0.317, 0.035 (x)	0.445, 0.050 (x)	2.281, 0.056	87.82, 1.33 (c)	2.152, 0.069	82.81, 1.95 (c)	238.2, 13.3 (x)	
18 000 ft+F	0.063, 0.003	2.549, 0.069	2.612, 0.071	0.116, 0.015	0.192, 0.019 (y)	0.308, 0.023 (y)	2.420, 0.075	92.55, 0.79 (d)	2.304, 0.067	88.17, 0.86 (c)	501.3, 29.4 (y)	

^aData expressed as mean, S.D. *n*=15 except for enamel [F] where *n* = 9. Units are mg/48 h per three rats, except for enamel [F].

^bSea level and 18 000 ft groups drank deionized water. Sea level+F and 18 000 ft+F groups drank water containing 25 ppm F.

Values in the same column with the same or no letter in parentheses are not significantly different. Intake, excretion, absorption, balance and enamel [F] of sea level and 18 000 ft groups were compared separately from the + F groups using the *t* test. The other variables were compared using ANOVA.

significantly lower in the hypoxic groups, indicating that absorption was higher than in the sea level groups. Overall, the total excretion of fluoride was substantially lower in the hypoxic groups so that the balances and fractional retentions were also greater. Consistent with these findings, the enamel fluoride concentrations were much higher in the hypoxic groups.

As noted in previous studies of this type (Angmar-Månsson & Whitford 1990), the appearance of the incisors of the rats in the hypoxic groups was severely disturbed regardless of the level of fluoride exposure. Grossly, the enamel was uniformly bleached to the colour of chalk and fractured in some areas so that the underlying dentine was exposed. Microradiographically, the enamel demonstrated severely disturbed mineralization patterns characterized by alternating layers of hypomineralized enamel and irregular surfaces similar to those seen in cases of severe fluorosis. These effects occurred in both hypoxic groups whose enamel fluoride concentrations were markedly different (Table 1). Thus, it was not possible to assess the effect of the higher enamel fluoride concentrations in the '18 000 ft+F' group. It was concluded, however, that chronic hypoxia promotes the retention of fluoride, resulting in higher fluoride concentrations in enamel and other tissues. It also adversely affects amelogenesis independently of the level of fluoride exposure.

Direct effects of fluoride on enamel

Three recent publications dealing with the effects of fluoride on enamel have described the possible mechanisms leading to fluorosis (Robinson & Kirkham 1990, DenBesten & Thariani 1992, Bawden et al 1995). Several of the reported effects on ameloblast proliferation, differentiation, ultrastructure, matrix synthesis and secretion have been observed only after extremely high fluoride exposures and/or appear too subtle to explain the changes in the quality of human enamel that typically occur when fluoride intake is only 1 mg/day or so during tooth development. Many *in vitro* studies have demonstrated fluoride effects on apatite nucleation, crystal growth and morphology but it is uncertain whether such effects are causally involved in the aetiology of fluorosis *in vivo* or whether they are secondary to other fluoride-induced effects. This does not necessarily mean that effects on cells and minerals are aetiologically unimportant but, at the present time, it does diminish the likelihood of their involvement in a causative way.

Fluorosed enamel is hypomineralized and contains more protein than normal enamel. As discussed above, however, the fluoride concentrations in transitional and early maturation enamel are relatively high. It is in these phases that the rates of removal of the protein matrix and the growth of crystallites in normal enamel increase dramatically. Further, these phases of amelogenesis appear to be the most sensitive to fluoride. For these reasons, a great deal of recent research has focused on the events occurring during these phases.

The growth of enamel crystallites is inhibited by a variety of proteins including high molecular weight amelogenins and others such as albumin. During the maturation of

normal enamel, all but the last traces of amelogenins and their fragments are removed. The persistence of protein in fluorosed enamel suggests an inhibition of enzymatic hydrolysis. Calcium-dependent serine proteinases from developing bovine enamel have been isolated, which suggests that high fluoride concentrations, through complexation of calcium, might inhibit their activity and the hydrolysis of amelogenins. Indeed, the activities of two serine proteinases (amelogeninases) were shown to be lower in fluorosed enamel than in normal enamel (DenBesten & Heffernan 1989). Aoba et al (1987a,b) reported the adsorption of high molecular weight amelogenins onto synthetic hydroxyapatite and inhibitory effects on crystal growth. Adsorption and growth inhibition did not occur when hydrolysis products of the proteins were tested.

The activity of the serine proteinases is greatest in developing enamel and, even in fluorosed enamel, the removal of amelogenins is nearly complete. Thus, it appears that the hydrolysis and removal of these proteins is delayed rather than diminished. The increased residence time of the high molecular weight amelogenins, however, may slow crystal growth sufficiently to affect ultimately the structural features of the mature enamel.

Another mechanism possibly involved in the development of dental fluorosis is the accumulation or retention of relatively high amounts of magnesium (Robinson 1984). Although the mechanism whereby magnesium concentrations increase in fluorosed enamel (or bone) has not been identified, it is known that the ion can inhibit the growth of apatite crystals and render them more soluble.

Acknowledgement

This work was supported by research grants DE-06113 and DE-06429 from the National Institute for Dental Research, National Institutes of Health, Bethesda, MD.

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DISCUSSION

Oldak: Do you have any direct evidence for the retention of amelogenins in the fluorosis cases?

Whitford: No, but we're currently looking at this.

Deutsch: In order to establish the sites where fluorosis originates, we have injected rats intraperitoneally with single doses of 2.5 mg fluoride, then killed them one to 30 days afterwards (Deutsch et al 1978). We also gave another group 100 ppm fluoride in their drinking water. We observed that fluoride was taken up preferentially at or just before the beginning of maturation. The major effect of fluoride on the appearance and on the chemical composition of enamel seemed to coincide with the preferential accumulation of fluoride at this stage. In addition, calculations based on the rate of incisor eruption indicated that the localized striations observed in the enamel 26 days after injection originated at this site.

Whitford: We observed that a single injection of fluoride affected the whole length of the tooth (Angmar-Månsson & Whitford 1985).

Fejerskov: In the mid-1970s it was thought that the period most sensitive to fluoride was the secretory stage, but subsequently it became more likely that fluoride instead affected protein degradation and mineral deposition during the maturation stage. Indeed, we have demonstrated that in wild roe deer, red deer and pigs, the maturation stage is affected rather than the secretory stage (Kierdorf et al 1993, 1996, Richards et al 1986).

I would also like to ask Janet Oldak whether the serine proteases could be candidates for the direct effect of fluoride.

Oldak: It is possible that fluoride affects the proteases by simply precipitating calcium ions (as CaF_2), which are required for the activity of these proteases. Recently, we reported the description of two major groups of enamel proteinases; the 'high molecular weight' and the 'low molecular weight' groups (Moradian-Oldak et al 1996a). In a series of *in vitro* experiments we have shown that the high molecular weight proteinase (60–68 kDa) cleaves the C-terminal segment of M179 amelogenin and it is a calcium-dependent metalloproteinase with an optimum pH of 8 (Moradian-Oldak et al 1994). Local pH changes in the enamel extracellular matrix may also influence proteinase activity and enamel maturation. Very recently we have shown that the serine proteinase ('ameloprotease-I'), which is responsible for amelogenin degradation during enamel maturation, is inhibited by EDTA, suggesting calcium dependency of the activity of that proteinase (Moradian-Oldak et al 1996b). There is some confusion in the literature regarding the definition of these proteinases because they are inhibited by EDTA and, therefore, some investigators reported them as being metalloproteinases. It is also likely that the serine proteinases are responsible for some forms of amelogenesis imperfecta because their malfunction would result in the retention of proteins, and particularly amelogenins.

Butler: How do you know they are serine proteases?

Oldak: We incubated the low molecular weight fraction, which was enriched in the 30 kDa proteinase, ameloprotease-I, with the M179 amelogenin substrate and observed the accumulation of the cleavage products only in the absence of serine proteinase inhibitors. It was extremely difficult to isolate any stable cleavage product for the identification of specific cleavage sites. We therefore suggested that further degradation of amelogenin was taking place because we have also observed products as small as 1 kDa. However, on the basis of amino acid analysis of the products accumulated we do know that the product region does not contain any tyrosine residues, indicating that the TRAP (tyrosine-rich amelogenin protein) sequence was cleaved from the N-terminus of the molecule probably prior to complete degradation.

I would also like to mention that the N-terminal sequence of ameloprotease-I (LPHVPHRIPPGYGRPXTXNEEGXNPYFFXXHG) matches that of the 32 kDa enamelin sequence reported by Tanabe et al (1990). Uchida et al (1991) have shown that the 89 kDa enamelin, for which the complete primary sequence has recently been reported, is the precursor of the 32 kDa protein (Fukae et al 1996). We have therefore identified the 32 kDa enamelin as being the enamel proteinase (ameloprotease-I) that is responsible for amelogenin degradation in maturing enamel. We propose that the 89 kDa enamelin, which is also derived from a 142 kDa enamelin, is a precursor for ameloprotease-I (Fukae et al 1996, Moradian-Oldak et al 1996).

Whitford: Presumably, there is a conceptual problem with the fluoride inhibition of these proteases by calcium precipitation in that calcium is present in the enamel at an extremely high concentration.

Oldak: Enamel is a calcified tissue and most of the calcium that is present in the tissue will probably be required for calcium apatite mineralization. It is possible that the cells can control the quantity of calcium in the extracellular fluid.

Whitford: I am aware that enamel is a calcified tissue. But the idea that you are presenting is that fluoride is inhibiting enzymatic activity by precipitating calcium and, in this way, limiting its availability to the enzymes. I assume that the calcium you refer to is not firmly bound in the mineral but, instead, is available to the enzymes in the enamel fluid in an ionic form. Because of the stoichiometry involved—that is the great excess of calcium over fluoride—I doubt that this is an important mechanism.

Elliott: Does 2 ppm fluoride have any biological effects on the morphology of other tissues?

Deutsch: Some years ago, when we were investigating the basic mechanisms associated with dental fluorosis, we were intrigued by the observation that levels of fluoride in plasma not much higher than normal levels (such as those occurring when 2 ppm fluoride is ingested during enamel formation) could cause the commencement of dental fluorosis but did not have any apparent visible effect on other soft tissues. By studying the distribution of fluoride along and across the different stages of development, we found that 'labile' fluoride accumulates preferentially in developing enamel just before or at the beginning of the maturation stage. Therefore, we suggested (Weatherell et al 1982) that the overlying enamel organ cells and the developing extracellular enamel matrix at this stage might be exposed to a much higher local concentration of fluoride than the other soft tissues.

Slavkin: The literature on the benefits of fluoride with respect to osteoporosis and other bone disorders is as yet controversial. American women are encouraged to ingest excess calcium, and if fluoride is beneficial, then ingesting excessive amounts of calcium, based upon Gary Whitford's work, would reduce the benefits obtained from fluoride. Therefore, we need to reconsider the clinical rationale for giving women calcium to retain bone mineral density.

Fejerskov: It is not only enamel that is affected by fluoride, but also cartilage and bone. Our methods of examining other tissues in the past have not been sensitive enough to demonstrate an obvious effect. Lyaruu et al (1987), using the *in vitro* tooth organ culture model, have shown that $0.5\mu\text{M}$ of fluoride added to culture media totally inhibits mineralization in the organ culture system, and that as soon as fluoride is removed mineralization starts again apparently undisturbed (see Melsen et al 1996 for review). Therefore, when discussing the effects of fluoride, we must remember to include general mineralization mechanisms to get the full picture.

Boyde: In this context, there is a direct relationship between parathyroid hormone secretion and the rate of bone cell differentiation and bone formation (Reeve 1996).

Mann: Colin Robinson showed that a transient increase in magnesium occurs around the transitional/maturation stages. Could this be related to the concentration of fluoride, i.e. from magnesium fluoride complexes?

Robinson: This is possible because there is a simultaneous increase in the levels of both magnesium and fluoride, which has always puzzled me because magnesium should increase the surface energy of the crystal surfaces. However, this fluoride is labile and one should not expect it to behave in the same way as when it is bound to the crystals. We've toyed with the idea that there is a magnesium phosphate/fluoride complex present at that stage, which would explain the curious labile behaviour of fluoride.

Simmer: I would like to discuss Gary Whitford's proposed mechanism for the reabsorption of fluoride from the kidney tubules in the presence of acidic urine. You gave a value of 5.6 for the pH of the urine, which is over two pH units away from the pK of fluoride (3.4). Therefore, less than 1% of fluoride is in a hydrogen fluoride state. Is it reasonable to conclude that this concentration of hydrogen fluoride accounts for the increased reabsorbance?

Whitford: Yes. Gutknecht & Walter (1981) found that the permeability coefficient of hydrogen fluoride across a lipid bilayer membrane was over a million times greater than that of ionic fluoride. We obtained similar results with a Sartorius-simulated gastric membrane system, which also has a lipid barrier.

Simmer: One would expect that result because hydrogen fluoride is not charged whereas fluoride ions are. But still only 1% of the total amount of fluoride would be in a state that could diffuse across the membrane when the pH is about 5.6.

Whitford: That's right, there is a small fraction of the fluoride in the urine in the form of HF at that pH. However, the charge and the large hydrated radius of the fluoride ion prevent it from crossing the epithelium. Also, remember that the permeability coefficient for hydrogen fluoride is very close to that of water itself.

Elliott: Rather than plotting a logarithmic pH function, should you not plot the uncharged hydrogen fluoride concentration? It is puzzling that there is a linear function above pH 5.5. I would expect the rate to be independent of pH above this value.

Whitford: It becomes independent of pH above about pH 6.5. Apparently, the concentration of hydrogen fluoride at lower pH values is sufficient to drive reabsorption from the tubules as the fluid column moves through the nephron.

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The role of enamel matrix proteins in the development of cementum and periodontal tissues

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Abstract. The role of Hertwig's epithelial root sheath (HERS) and of the enamel-related proteins in the development of acellular cementum are reviewed. The inner layer of HERS is an apical extension of the ameloblastic layer in the crown. A number of studies now indicate that the cells of HERS have a secretory stage similar to the ameloblasts. In rats and mice the secretory product of the HERS cells does not seem to be amelogenin, which is the main protein of the enamel matrix. In humans, however, amelogenin has been demonstrated at the apical ends of the roots of developing teeth. The development and distribution of coronal cementum in various species are discussed. The amelogenins have been remarkably well conserved between species. Experiments in monkeys have shown that it is possible to induce formation of acellular cementum by application of porcine enamel matrix on a denuded root surface, which thereby promotes periodontal regeneration. These results further support the idea that enamel-related proteins are involved in cementum formation.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 246-260

Hertwig's epithelial root sheath (HERS) plays an important role in the development of the dental roots by inducing the differentiation of odontoblasts and the formation of the first peripheral layer of dentine (Selvig 1963, Ten Cate 1978, Owens 1980, Thomas & Kollar 1989). For a long time, it was believed that this was the only function of HERS and that the subsequent development of cementum was induced by exposure of the newly formed dentine to the mesenchymal cells of the dental follicle (Armitage 1986, Cho & Garant 1988, Bosshardt & Schroeder 1991). However, recombination experiments between slices of root dentine and follicular cells have shown that an exposed dentine surface is not a sufficient stimulus for cementoblast differentiation (Thomas & Kollar 1988).

A number of studies now indicate that HERS, or its products, also participates in the formation of cementum. The inner layer of HERS represents an extension of the ameloblast layer in the crown, and Slavkin & Boyde (1975) and Slavkin (1976) proposed that enamel-related proteins from HERS initiated the formation of

acellular cementum. Schonfeld & Slavkin (1977) showed that enamel matrix proteins were formed on the developing root-analogue surfaces of rabbit incisors. This observation was supported by light and electron microscopic studies by Owens (1978, 1980), who found that cells of HERS in developing rat molars exhibited organelles suggestive of a secretory activity. Further support was gained from scanning electron microscopic and autoradiographic studies in monkeys (Lindskog 1982a,b, Lindskog & Hammarström 1982). These studies showed that the inner layer of HERS has a secretory stage and that an enamel-like material is formed on the root surface prior to the formation of cementum or as an initial step in this process. In subsequent years, Slavkin and co-workers (Slavkin et al 1988, 1989) showed in a number of studies that some proteins in acellular cementum are immunologically related to amelins and amelogenins. However, Thomas et al (1986) were not able to detect enamel proteins in this location. *In situ* hybridization studies by Luo et al (1991) on the developing mouse molar indicated that the enamel-related proteins postulated to be synthesized by HERS were not identical to amelogenin. In agreement with the studies by Luo et al (1991), we have been unable to demonstrate any expression of amelogenin in the cells of HERS in rats by means of *in situ* hybridization (Fong et al 1996). However, we have found that another, recently identified, enamel-related protein, called ameloblastin (amelin) is expressed by the epithelial cells during root formation in rats (Fong et al 1996). Immunohistochemical studies with rabbit antibodies towards porcine amelogenin did not result in staining of any tissue at the apical forming root ends of rat molars. In contrast, there was a distinctly stained layer at the surfaces of the apical ends of the roots of developing human teeth (L. Hammarström, unpublished data 1995). There seem to be differences between the development of the roots of murine and human teeth.

An association between enamel matrix secretion and cementum formation should not be surprising, since so-called coronal cementum is present on the enamel surface in a number of mainly herbivorous species (for review see Schroeder 1985). The coronal cementum is usually of the acellular type and its distribution may vary between different species. In guinea pigs acellular cementum occurs as plaques or patches in fenestrations of the covering ameloblastic layer (Fig. 1) (Hunt 1959). Sheep and rabbits have a more complete coverage of the enamel by acellular cementum (Fig. 2). In humans also, acellular cementum has been found to cover the enamel to a varying extent (Silness et al 1976, Schroeder 1985). In cases of amelogenesis imperfecta, acellular cementum has been found in areas where the ameloblastic layer is absent (Weinmann et al 1945, Listgarten 1967). Experimental studies have shown that cementum, mainly of the acellular type, is formed on the surface of the developing enamel of mouse molars after removal of the covering enamel organ (Heritier 1982). We have repeated the studies by Heritier (1982) in rats and confirmed his results (Fig. 3).

Another interesting link between the secretion of enamel matrix and the formation of acellular cementum has been demonstrated in studies on the effects of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) on developing teeth. HEBP is a

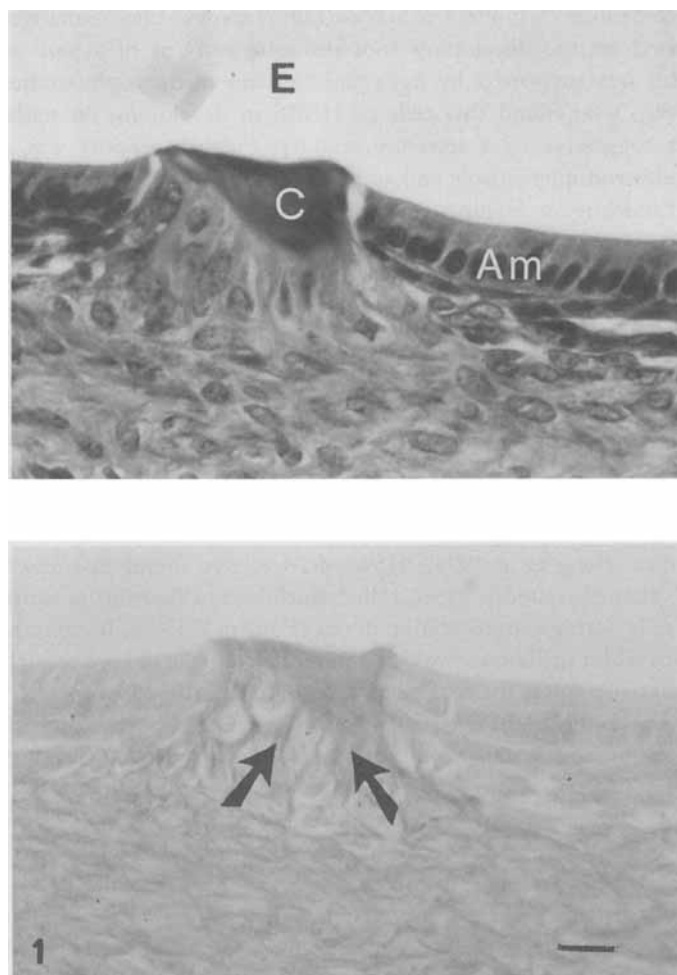


FIG. 1. Part of a guinea pig molar showing a cementum pearl or plaque in a small fenestration of the ameloblastic layer. Haematoxylin and eosin (upper) and van Gieson (lower) stainings. Note the extending collagenous fibres (arrows). Am, ameloblasts; C, cementum; E, enamel space. Bar = 20 μ m.

bisphosphonate that inhibits both mineralization and resorption of bone and it is used in the treatment of certain diseases of bone. When single or multiple injections of HEBP were given to young rats or mice, the initial mineralization of the mantle dentine was delayed or inhibited and neither enamel matrix nor acellular cementum was formed on top of the non-mineralized dentine (Fig. 4) (Beertsen et al 1985, Josephson et al 1990, Alatli et al 1994). Possibly the effects of this drug on cementum

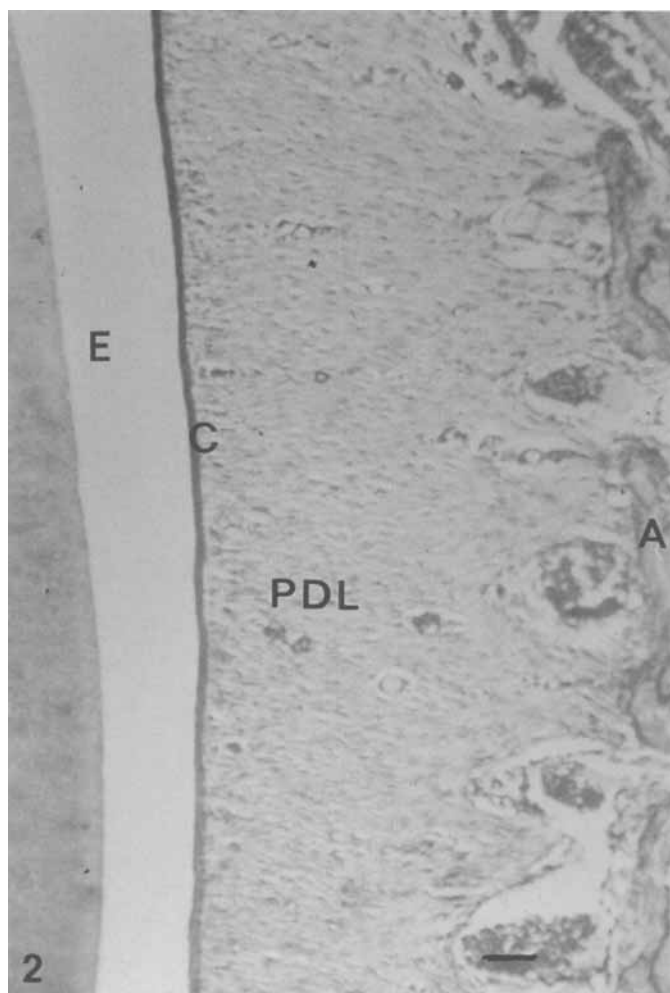


FIG. 2. Part of a rabbit molar showing a thin layer of cementum that covers the enamel surface. Haematoxylin and eosin staining. A, alveolar bone; C, cementum; E, enamel space; PDL, periodontal ligament. Bar = 20 μ m.

formation were secondary to the inhibition of the secretion of enamel-related proteins by HERS.

The major proteins of the enamel matrix are known as amelogenins. They have been remarkably well conserved between species (Brookes et al 1995). Against this background, it was suggested that enamel matrix from one species may induce cementum formation in another. A number of studies on monkeys were carried out to test this hypothesis (L. Hammarström, L. Blonlof & S. Lindskog, unpublished

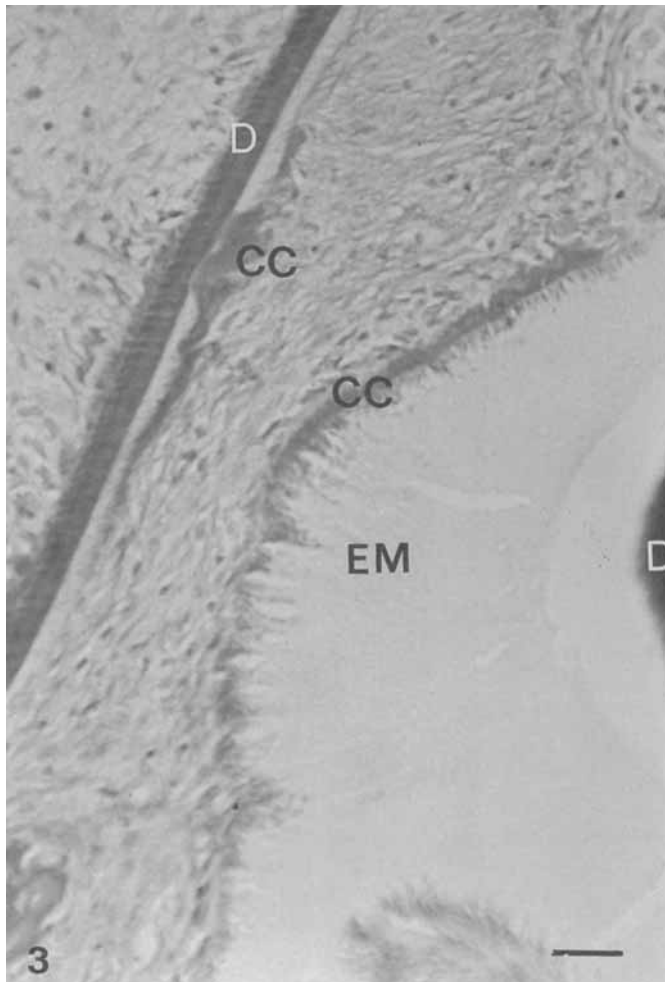


FIG. 3. Developing rat molars from which the covering ameloblastic layer has been removed. Ten days after the removal of the ameloblasts the denuded areas of the enamel matrix are covered by a layer of 'coronal cementum'. Haematoxylin and eosin staining. CC, coronal cementum; D, dentine; EM, enamel matrix. Bar = 50 μ m.

data 1986). These studies showed, that after the application of porcine enamel matrix proteins onto a denuded dentine surface, acellular cementum was formed with anchoring periodontal fibres (Fig. 5). In addition to the regeneration of the cementum, new alveolar bone was formed. In control experiments in which no enamel matrix was applied, minimal amounts of cementum and alveolar bone were formed. These results support the idea that enamel-related proteins are involved in the formation of acellular cementum.

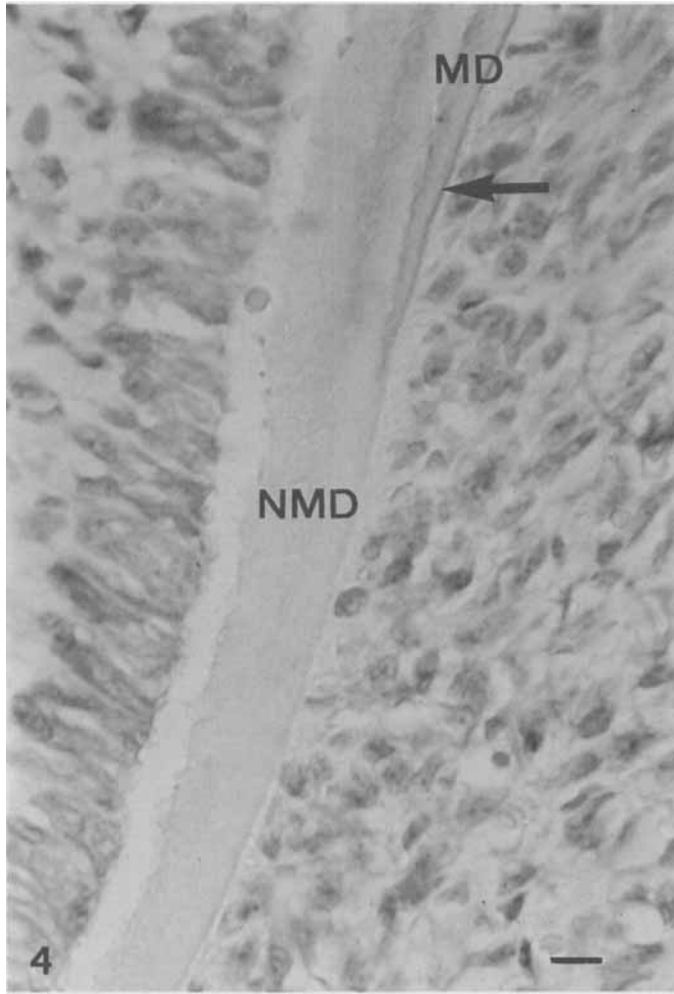


FIG. 4. Part of the developing root of a rat molar, two days after a single injection of 1-hydroxyethylidene-1,1-bisphosphonate (dose corresponding to 10 mg P/kg body weight), which inhibits both mineralization and resorption of bone. No acellular cementum was formed on the non-mineralized dentine. Haematoxylin and eosin staining. MD, mineralized dentine; NMD, non-mineralized dentine. Arrow indicates acellular cementum. Bar = 20 μ m.

During tooth development several epithelial–mesenchymal interactions have been found to occur. Such interactions may require direct intercellular contacts between heterologous cell sheaths, whereas others may be mediated via the extracellular matrix or by diffusible growth factors (Slavkin 1979, Gurdon 1992). The induction of acellular cementum formation by enamel matrix proteins seems to be an example of cell–matrix interaction. However, so far no amino acid sequence specific for cell

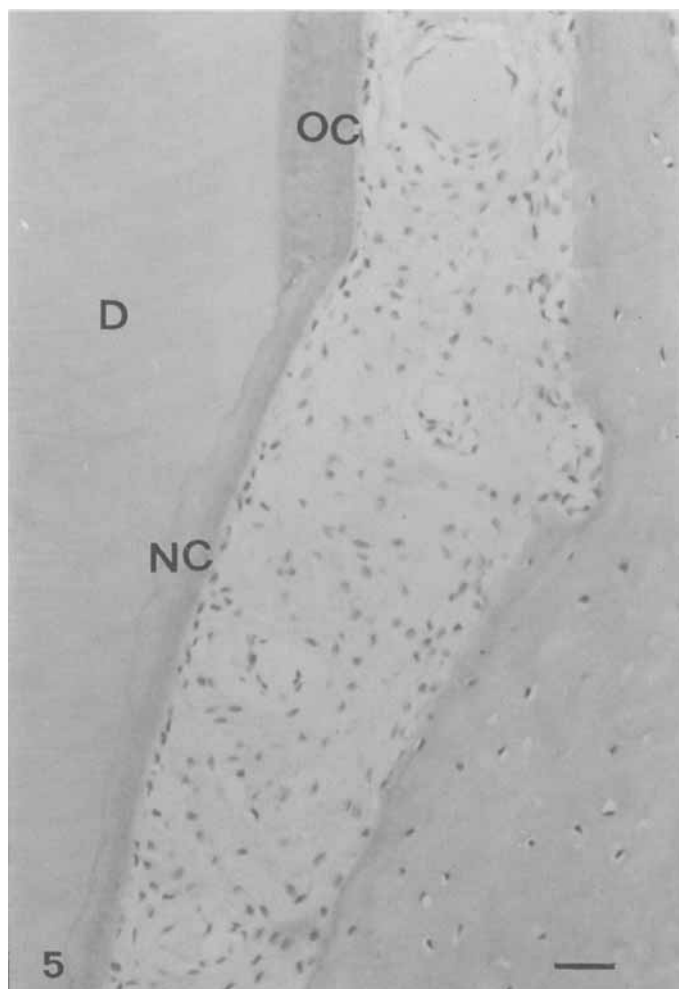


FIG. 5. Experimental cavity in the root of the lateral incisor of a monkey. The incisor was gently extracted and the experimental cavity was made by means of a round burr under constant irrigation with physiological saline. Porcine enamel matrix was then applied in the cavity, after which the incisor was immediately replanted. Eight weeks after the replantation, the cavity was covered with a layer of acellular cementum. Haematoxylin and eosin staining. D, dentine; OC, original acellular cementum; NC, new, regenerated cementum. Bar = 50 μ m.

attachment has been identified in amelogenin. The formation of both cementum and new alveolar bone is in agreement with studies on the morphogenesis of the dental tissues showing that the alveolar bone has its origin in the dental anlage and that the integrity of the alveolar bone is maintained by cells at the root surface (Freeman & Ten Cate 1971, Andreasen 1981).

The treatment of advanced periodontitis is gradually changing from conservative procedures, with the limited aim of halting the progress of the disease, to a more offensive approach aiming at the regeneration of the lost periodontal attachment. Examples of such new treatment modalities are guided tissue regeneration, the use of bone grafts, and the application of various growth factors and attachment proteins. The reader is referred to recently published reviews of such procedures (Brunsvold & Mellonig 1993, Caffesse & Quinones 1993, Greenstein & Caton 1993, Gottlow & Nyman 1996, Howell et al 1996). The experiments with the application of enamel matrix proteins to the root surface showed that it was possible to mimic the normal development of cementum and in this way induce regeneration of acellular cementum, periodontal ligament and alveolar bone. A new treatment procedure for periodontal regeneration, based on the use of an enamel matrix preparation (EMDOGAIN® BIORA AB, Malmö, Sweden), is now being launched for clinical use. An extensive description of experimental and clinical results will be published as a supplement to the *Journal of Clinical Periodontology*.

Acknowledgements

I am very grateful to Idil Alatlí and Dan Fong for their comments on this manuscript. These studies were supported by the Stockholm County Council and the Swedish Medical Research Council (Grant No. 06001).

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DISCUSSION

Winter: When you are talking about cementum, are you referring to acellular cementum?

Hammarström: Yes. The cellular cementum may, at least in the rat, have a developmental pattern that differs from that of acellular cementum. The epithelial cells that are present at the surface of the acellular cementum stay together in clusters. In the cellular cementum the inner and outer layers of the epithelial root sheath cells seem to have separated. The inner layer of epithelial cells remain at the inner surface of the cellular cementum, whereas the outer layer of cells is found at the outer surface of the newly formed cellular cementum. We have also found that a single injection of a bisphosphonate, the name of which is often abbreviated to HEBP (1-hydroxy-ethylidene-1,1-bisphosphonate), given at an early stage of root formation permanently inhibits the formation of acellular cementum in rat molars. Instead, a cellular hard tissue matrix is formed on the radicular dentine surface. This hard tissue matrix contains epithelial cells both at the inner surface towards the dentine and at the outer surface in the same way as the normal cellular cementum. From studies on the early development of the dentine we know that the outer layer of dentine is induced by an epithelial-mesenchymal interaction between the inner layer of the epithelial root sheath and the mesenchymal cells of the dental papilla. Our hypothesis is now that the cellular cementum matrix is formed in a similar way, i.e. by exposure of the mesenchymal cells of the dental follicle to the epithelial cells of the inner layer of the root sheath.

Nanci: Dieter D. Bosshardt, a postdoctoral fellow in my laboratory, has recently obtained results that support those of Lars Hammarström. We chose to focus on pigs and humans because the formation of the root and cementum in these species takes place over a relatively long period and, therefore, cellular changes and accumulation of matrix proteins also occurs over a longer period of time. Hence, proteins expressed

at low levels may accumulate over time to concentrations detectable by immunocytochemistry. In both human and porcine teeth immunoreactivity for enamel proteins was observed along the root surface using an antibody to porcine amelogenins (courtesy of Hardy Limeback) and/or an anti-24 kDa rat amelogenin antibody we prepared using the chicken egg yolk system. The labelling was focal and not present over the entire root. In porcine roots in which Hertwig's epithelial root sheath (HERS) cells were just beginning to disaggregate, the material immunoreactive for amelogenin was clearly associated with cells that had epithelial characteristics. Banded collagen was also observed in close association with these epithelial cells and enamel proteins. These results suggest that HERS cells produce collagen and some enamel protein on top of the root dentine and contribute, at least initially, to cementum formation.

Jones: What is the evidence that collagen is secreted from the epithelial cells?

Nanci: We have no direct evidence that it is. The position of the collagen fibrils and enamel proteins with respect to the epithelial cells strongly suggests that they are responsible for the production of these extracellular matrix proteins. However, one cannot rule out a contribution from the mesenchymal cells in close proximity to the HERS cells. In the regenerating fish scales of *Calamoichthys calabaricus* one also finds banded collagen fibrils and amelogenin between epithelial cells and a mineralized collagenous matrix. In this case, there are no mesenchymal cells beyond the epithelial cells and the collagen must have originated from them.

Veis: Have you stained the cells with an anti-collagen antibody?

Nanci: No, this has to be done. However, in some areas, the enamel proteins and collagen on the root surface are separated from mesenchymal cells by several layers of cells. Indeed, like the enamel organ, these cells appear to seal their products against the dentine surface. In this case, it is difficult to conceive how mesenchymal cells several micrometres away could be responsible for the production of the banded collagen associated with the epithelial cells. Furthermore, it is well established that certain epithelial cells can produce collagen type I (Trelstad & Slavkin 1974, Sugrue & Hay 1986, Hay 1991). There is also enamel protein antigenicity at the interface between the junctional epithelium and the tooth surface. The internal basal lamina of the junctional epithelium labels with antibodies to amelogenin (Nanci et al 1995). This layer blends in cervically with afibrillar acellular cementum, which is rich in osteopontin and bone sialoprotein. The amelogenin antigenicity detected in the junctional epithelium does not represent residual enamel proteins. Indeed, amelogenin antigenicity is also detected at the interface between the root surface and regenerating junctional epithelium following surgical ablation of part of the gingiva. This observation indicates that the newly differentiated junctional epithelium cells can produce enamel-like proteins. Hence, inner enamel epithelium-derived cells, other than ameloblasts, can produce enamel protein-related matrix constituents.

Oldak: You used the term 'enamel protein' and I assumed that you were referring to amelogenin. Have you looked at the other enamel proteins?

Nanci: As some of the amelogenin antibodies we use recognize several protein bands on immunoblots of enamel, we prefer to use the more general term 'enamel proteins'. We now have access to antibodies against ameloblastin, ameloprotease I and tuftelin, and we intend to repeat our immunolabelling experiments with them. Furthermore, we hypothesize that some inner enamel epithelial cells undergo epithelial-mesenchymal transformation, and these cells would then produce 'typical' mesenchymal products, such as osteopontin and bone sialoprotein. Indeed, this is what we see at the enamel-free area on rodent molars (Bosshardt & Nanci 1996). I believe that Maggie Zeichner-David has similar observations on immortalized HERS cells *in vitro*.

Fejerskov: In 1979 we had a paper returned from *Anatomical Record* because we would not eliminate our illustrations of collagen fibres that were located between ameloblasts and the enamel-free cusp area. We interpreted these as being produced by maturation ameloblasts covering the enamel-free cusp of the rat molars.

Zeichner-David: Antonio Nanci is correct. We have been working with an immortal HERS cell line obtained from mice. When we subject these cells to conditions that promote differentiation they undergo an epithelial-mesenchymal transformation and synthesize collagen. We have demonstrated this by reverse transcriptase PCR analysis and by using antibodies against collagen type I. They also end up producing a mineralized cementum-like extracellular matrix, although I am reluctant to call it cementum because we don't really know what cementum is. In the beginning, these cells synthesize enamel proteins such as tuftelin and ameloblastin but we are unsure as to whether they also synthesize amelogenin. We only get a positive PCR result when we use primers from exons 6 and 7; and there is a small amount of antibody staining, but when we extract the proteins we do not observe any amelogenin proteins on a Western blot. The cells also make some dentine components, such as dentine matrix protein and dentine sialoprotein.

Winter: Which type of collagen do they produce?

Nanci: Collagen type I.

Slavkin: In the early 1970s Bob Trelstad and Elizabeth Hay published an interesting paper on corneal collagen (Trelstad et al 1973). They used metabolic labelling coupled with column chromatography and demonstrated that $\alpha 1$ and $\alpha 2$ type I, and type IV collagen chains were synthesized and secreted by the epithelial cells of the cornea. Subsequently, Bob Trelstad and I did a similar experiment with the epithelium and mesenchyme of embryonic rabbit teeth. We found that the enamel organ epithelium synthesized and secreted both type I and type IV collagen (Trelstad & Slavkin 1974).

Thesleff: We have also used *in situ* hybridization to show that the epithelial cells in the tooth germ express type I collagen (P.-L. Lukinmaa, unpublished observations 1995).

Fejerskov: We published in the early 1970s that occlusal fissures are covered by afibrillar, acellular cementum in humans (Silness et al 1976), which fits nicely with the concepts that the enamel organ cells produce this type of cementum.

Thesleff: Do you have any evidence of growth factor activity associated with the matrix?

Hammarström: We have looked for growth factors by means of various immunological methods such as IRMA, RIA and ELISA, but we have not found any. We have tested for at least 20, including basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor 1 and 2, platelet-derived growth factor, interleukins 1, 2, 3, 4, 5 and 6, granulocyte macrophage colony-stimulating factor, γ -interferon, transforming growth factor β , tumour necrosis factor, fibronectin and calbindin.

Nanci: I agree that it is unlikely that growth factors are present in the enamel protein preparations. However, we have obtained similar results to those of Lars Hammarström with a growth factor cocktail in a porcine model, i.e. that bone, cementum and junctional epithelium repair were improved and accelerated.

Slavkin: The limitation with this approach is that it tests only those growth factors that we know of. Another approach would be to employ methodologies such as chemotaxis and differential display PCR techniques to possibly identify new gene products.

Thesleff: The nanosphere structure may have a role. Alternatively, other matrix molecules that act via cell surface receptors (such as integrins) may be coupled to the growth factor signalling system.

Hammarström: Yes, we believe that a cell-matrix interaction plays an important role.

Veis: When you close the flap after you put the implant in, how do you prevent blood from entering?

Hammarström: I don't exclude anything. Blood can enter the system.

Veis: Therefore, even if growth factors are not initially present the matrix could contain chemoattractants that attract cells or growth factors from the blood. This would mean that the amelogenin factors have no special 'differentiating' activity, except for possibly being chemoattractants.

Mann: Have you looked at the ultrastructure in detail?

Hammarström: No, we have not looked at this.

Diekwisch: Are you sure that the enamel proteins in the gel are responsible for the formation of new cementum rather than other components of the gel matrix?

Hammarström: We have carried out studies with the vehicle gel as control and no cementum was formed. As a matter of fact, we have tried a number of different vehicles and cementum was formed to an appreciable degree only in the cases when enamel matrix was used without a vehicle or when it was used with propylene glycol alginate as a vehicle (the propylene glycol alginate vehicle left the application site shortly after the application).

Diekwisch: Collagen gels, which have been recently used in guided-tissue regeneration, also result in an increased bone and cementum formation. Also, your immunohistochemistry results on frozen sections of human teeth did not convince me that there were enamel proteins in the epithelial cells. I raise this doubt because during mammalian tooth evolution there are many cases where cementum is

produced on enamel. The real issue of interest is to determine whether the enamel proteins are important for the deposition of cementum, or whether cementum is just secreted on top of a layer rich in enamel protein remnants similar to the secretion of cementum on top of dentine.

Simmer: How far does enamel extend down the root? And secondly, what does the histology show at time points nearer to the implantation of the enamel proteins? Do you observe a significant immune reaction?

Hammarström: The deposition of enamel between dentine and cementum is not observed in all teeth and it doesn't extend over the entire root region. We believe that the amounts of enamel matrix deposited on the dentine surface may vary and possibly also its degradation and mineralization. The highly mineralized enamel under the cementum is most commonly found in the cervical third of the teeth.

In the monkeys that were killed eight weeks after application there were no signs of any local immune reaction. We have not been able to follow in detail the changes of the tissue reactions with time.

Snead: Rodent cementogenesis does not seem to proceed in the same way as human cementogenesis. Is it possible, therefore, that there are many pathways that achieve the same result?

Hammarström: I don't know.

Boyde: There are many types of rodent, so one should not make generalizations from one type of rodent. Moreover, in the rat molar root epithelial rests are incorporated between the dentine and the cementum (Lester & Boyde 1970), but this does not occur in the incisor. Therefore, even within one animal two different processes are occurring.

Nanci: Root and cementum formation occur more quickly in small rodents than in humans. Therefore, if epithelial-mesenchymal transformation occurs, there will be comparatively little time for the enamel proteins to be produced and accumulate extracellular as the cells go from an epithelial to a mesenchymal phenotype.

Hammarström: I would finally like to caution against using the term epithelial-mesenchymal transformation until we know with more certainty if such a process takes place in the root sheath cells.

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The biomimetics of enamel: a paradigm for organized biomaterials synthesis

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Abstract. The formation of enamel takes place through a sequence of processes that can be mimicked in inorganic materials chemistry. This chapter describes the generic features of enamel biomineralization in terms of a house-building analogy. Four stages are identified: supramolecular preorganization and spatial patterning; interfacial molecular recognition in inorganic nucleation; vectorial crystallization; and pattern evolution and hierarchy. Each of these concepts can be translated into synthetic approaches to the formation of inorganic materials with organized architectures. An example of applying this biomimetic paradigm is described. Supersaturated water-in-oil microemulsions have been used to synthesize microskeletal calcium phosphates by controlled nucleation and vectorial growth in constrained reaction environments. The results of these preliminary studies suggest that biomimetic concepts could be useful in the fabrication of biomaterial implants with controlled porosity and microstructure.

1997 Dental enamel. Wiley, Chichester (Ciba Foundation Symposium 205) p 261–274

Although enamel is an unusual biomineralized tissue—notably in respect of the enormous aspect ratio of the spaghetti-like carbonated apatite crystals, and the exceedingly high mineral volume fraction of the mature biomaterial—many of the underlying processes accountable for its formation can be accommodated in a generic description of biomineralization. This chapter addresses how these general features of enamel biomineralization can be conceptually linked to new strategies in the synthesis of inorganic materials with organized architectures.

I begin with a discussion of enamel biomineralization as if we were constructing a house, from the preparation of the building site, through laying of the foundations and constructing a frame, to filling in the bricks and mortar and organizing higher-order networks. This metaphor will allow us to highlight the chemical steps required for construction, as well as establishing potential mechanistic archetypes that can be explored in biomimetic routes to self-assembled calcium phosphates. What our discussion will lack is a focus on the genetic and metabolic codes that provide the pattern formation, differentiation and tissue specificity. This is a serious defect: in our house-building analogy, it is like forgetting to employ an architect!

There are many ways of going about building a house; for example, building directly on site, as against bringing in prefabricated units. Interestingly, both these strategies have biological counterparts. As we will see, the 'enamel house' is conventional in that biominerals are generated *in situ*, but unusual in that the building site undergoes time-dependent changes in composition. Indeed, enamel biomineralization is almost unique in that not only can we speak of bioinorganic morphogenesis but we can also consider the process to involve programmed changes in composition and form — bioinorganic metamorphism. This aspect of enamel maturation is a striking example of the importance of synergism in biomineralization (Fig. 1). That is, control of the physical processes of crystallization is coupled in time and space with the patterns of tissue-specific developmental programmes involving regulatory elements such as growth factors and homeotic gene transcripts. The generally accepted view, if not explicitly stated, is that everything is under genetic and metabolic control, and that inorganic crystallization is subsumed within this morphogenetic programme. Whilst this is undoubtedly a sensible paradigm, it would be remiss not to suggest that at least one additional regulatory switch might be activated by the biomineral itself. For example, the formation of apatite involves a chemical reaction ($\text{Ca}^{2+} + \text{HPO}_4^{2-} \rightarrow [\text{CaPO}_4] + \text{H}^+$) and the local pH of the solution adjacent to the growing tips of the filamentous enamel crystals might be significantly lower than at the non-growing sides of the same crystals positioned closer to the dentine–enamel junction (DEJ). This might be one trigger for the concomitant assembly of amelogenin nanoaggregates behind, but not at the growth front (amelogenins are more soluble at $\text{pH} < 7$). Another possibility is that the release of protons is coupled to Ca^{2+} efflux from the adjacent ameloblasts by antiport transport through a $\text{Ca}^{2+}/2\text{H}^+$ membrane pump.

The enamel house

The four stages of building the enamel house are summarized in Table 1. Enamel is constructed within an elaborate building site that is orchestrated by the ameloblasts. In essence, the positioning, secretion and movement of these cells generates a spatially organized reaction environment for mineralization. However, unlike many biominerals, this site is not preformed, but continually generated and reconstructed by the activity of the ameloblasts (Lowenstam & Weiner 1989).

Initial development of the building plot resides with the interfacial activity of the ameloblasts. Structurally, the site is delineated between the mineralizing bedrock of the DEJ and the ruffled cell membranes (Tomes' process) of the retracting ameloblasts (Weiner 1986). Together, they provide the boundary conditions for construction of the extracellular protein matrix comprising the hydrophobic amelogenin proteins and aggregates, and the hydrophilic enamelines. Like many biomineralization processes, the building of an organic precursor material from the facilitated assembly and self-assembly of biomolecular constituents represents the initial stage of development (Mann 1993).

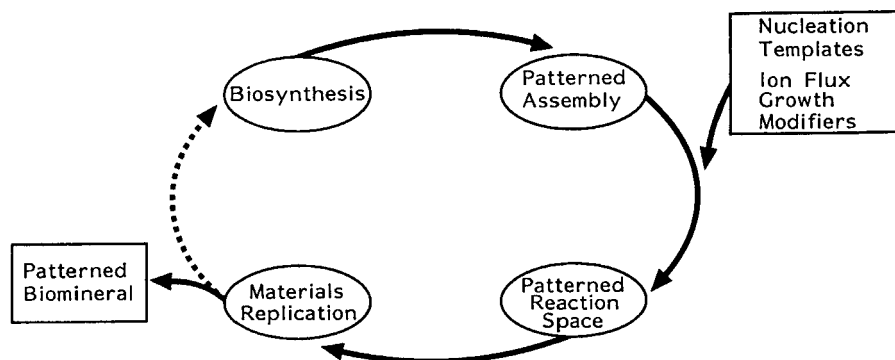


FIG. 1. Scheme of pattern formation in biomineralization. Biosynthesis results in the patterned assembly of organic supramolecular architectures that are functionalized for mineralization by incorporation of proteins and other macromolecules involved in nucleation and growth. Mineralization within the patterned space produces an inorganic 'replica' of the preorganized organic assembly. This may be a one-step 'cast in a mould' transcriptive process or it may involve a series of synergistic steps (dashed line) resulting in morphogenesis.

TABLE 1 The four stages of construction of enamel and associated biomimetic principles

<i>Building process</i>	<i>Function</i>	<i>Enamel house</i>	<i>Biomimetic principle</i>
Building site	Delineated reaction environments	Dentine–enamel junction/Tomes' process/matrix	Supramolecular preorganization and spatial patterning
Foundations	Site-specific inorganic nucleation	Dentine–enamel junction/enamelins?	Interfacial molecular recognition
Assembly (bricks and mortar)	Crystal growth and termination	Ameloblast assembly? Amelogenin retraction	Vectorial crystallization
Networking	Higher-order architectures	Biochemical and cellular processing	Pattern evolution/hierarchy

With an initial building site established, the next stage in constructing the enamel house is to lay the inorganic foundations. This requires a precise plan of the positional and structural information required for crystal nucleation. The general consensus in biomineralization is that chemical functionalization of preorganized organic supramolecular assemblies can be translated into the ordering of crystal nuclei (Mann 1988). How this occurs in enamel is not clear. Perhaps the initially secreted enamelins are ordered into periodic structures at the DEJ. If so, repeat distances between binding sites, and side group functionalities complementary to the formation of c -axis-oriented crystals (possibly of octacalcium phosphate), must be expressed at the organic surface. Alternatively, aperiodic assemblies of enamelins might be sufficient to initiate

nucleation at localized centres at the DEJ surface, with the c -axis orientation being determined by intrinsic crystallographic properties—for example, this axis is often the direction of fast growth in apatite crystals. Could the collagen of dentine play a role in these putative organizational processes (Arsenault & Robinson 1989)?

Once nucleated, the calcium phosphate crystals grow within the soft mineralized tissue of immature enamel to enormous lengths (up to perhaps 100 μm). At the same time, the widths and thicknesses of these crystals are restricted to nanometre dimensions such that the immature tissue consists of a framework of oriented ribbon-shaped inorganic strands (10–20% by volume) embedded in an (organized?) organic gel (Robinson et al 1983). The vectorial growth process resulting in these inorganic nano-ribbons is established by the highly directional movement of the ameloblasts away from the mineralization front. The patterning of the crystallization field is highly regulated, probably by maintaining a distance of only a few tens of nanometres between the ends of the crystals and the cell membrane. Together, by coupling crystallization and cell movement, a hybrid inorganic–organic composite gel is built on a macroscopic length scale.

With an inorganic frame in place, the final stage of enamel biomineralization involves a major reconstruction of the building site. In particular, whereas the enamelines remain associated with the apatite crystallites, the amelogenins are degraded and removed from the tissue, being replaced by water and supersaturated calcium phosphate solution (Robinson et al 1983). This results in a thickening of individual crystallites and lateral coalescence. The transformation of what was basically an organic gel to almost pure inorganic mineral (90% mineral by volume) by cellular and biochemical flow processing is remarkable.

The programmed changes in the structural and chemical properties of maturing enamel result in changes in the biomineralization pattern such that higher-order architectures are constructed. The enamel house is not only hardened but also organized into an array of 5 μm diameter rods each containing hundreds of the oriented filamentous crystallites. Whereas the patterning of the individual crystallites is associated with the size and positioning of the Tomes' process, that of the interweaved rods is commensurate with the dimensions of individual ameloblasts and their time-dependent spatial locations (Weiner 1986). Changing from one patterning agent to another in the course of enamel maturation is of profound importance as an idea in biomimetic materials chemistry because it suggests that hierarchical inorganic materials might be synthesized in an analogous manner in 'one-pot' chemical systems. Indeed, if similar processes can be induced in synthetic systems it should be possible to fabricate inorganic materials that 'evolve' in well-defined patterns according to the chemical and structural status of their associated reaction environments.

Biomimetic synthesis

Can we translate the underlying principles of enamel biomineralization into synthetic strategies for the chemical construction of organized inorganic materials? There are

some key points of connection which suggest that a biomimetic approach is feasible (Mann 1995) (Table 1). Firstly, there are a number of synthetic counterparts of the organized organic matrix. For example, organic supramolecular assemblies—such as surfactant micelles and microemulsions, lipid vesicles, helical ribbons and cylinders, and liquid crystalline gels—can be synthesized with well-defined micro-architectures. Secondly, nucleation within these biomimetic matrices can be promoted by judicious chemical modification of functional headgroups so that ion binding, and redox and nucleation sites are built into the supramolecular assembly. For example, Langmuir monolayers of surfactant molecules often behave very specifically in this manner (Heywood & Mann 1994). Geometric, stereochemical and electrostatic interactions are principal features of molecular recognition at these inorganic–organic interfaces.

Thirdly, vectorial crystal growth can be achieved by designing synthetic systems with shaped architectures—rods, tubes, or interconnected networks and strings of vesicles, for example—although an attempt to attain the enormous aspect ratio of enamel crystals is probably over-ambitious at the present time. And finally, certain mixtures of oil, water and surfactants can undergo phase changes with modifications in temperature, pH and ionic strength, suggesting that the time-dependent properties of the enamel matrix might be superficially mimicked by crystallization reactions in media similar to that of vinaigrette salad dressing!

Indeed, it turns out that certain mixtures of oil, water and surfactant fulfil most of the above biomimetic properties. In general, an oil phase can be stabilized in a water phase, and vice versa, by the addition of surfactants that partition at the oil–water interface. Depending on the composition of the mixture, the thermodynamically stable state consists of oil droplets in water (micelles) or water droplets in oil (reverse micelles). These may be less than 5 nm in diameter although droplets with dimensions of several tens of nanometres can be stabilized in microemulsions. Now, in a few specific cases, unusual microstructures, called bicontinuous microemulsions, are formed. As their name suggests, these mixtures do not contain discrete entities, such as droplets, but consist of two continuous structures—one of oil, the other of water. The envisaged microstructure is shown in simplified form in Fig. 2. Each component is present as an elaborate meshwork of nanometre-sized channels that are stabilized by the surfactant. Furthermore, the water and oil networks are interpenetrating such that the mixture is optically clear. Finally, the channels are rapidly fluctuating, disconnecting and reforming but these time-scales are short compared with measured bulk properties such as ionic conductivity.

The objective was to promote calcium phosphate crystallization in this organized reaction media such that the inorganic material is confined to the framework of interconnected water channels. Moreover, the surfactant headgroups point into the water phase, so there is the additional possibility of inducing site-specific nucleation at the charged surface. If the inorganic pattern produced by mineralization is generated as a direct replica of the microemulsion structure then a calcium phosphate ‘nanoskeleton’ should be obtained. Alternatively, the crystals could nucleate in the

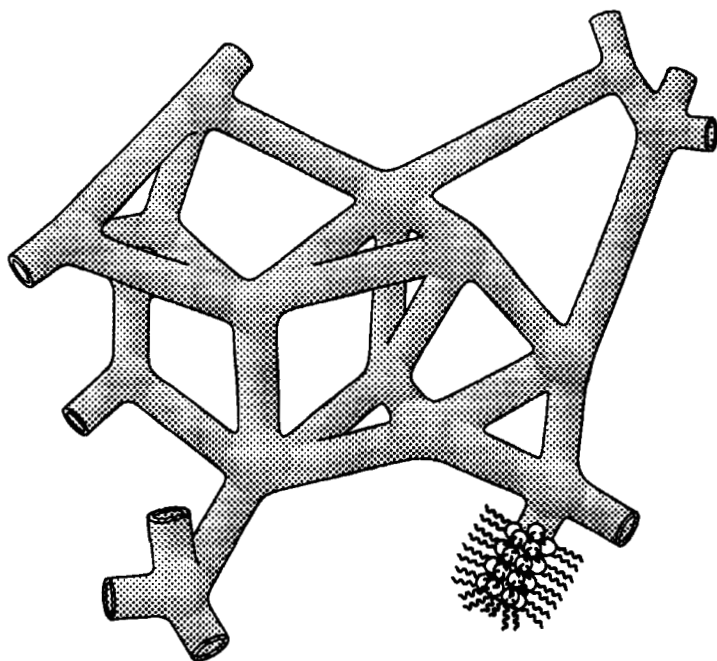


FIG. 2. Schematic drawing of a bicontinuous microemulsion showing the interconnected water channels within an oil matrix. The surfactant molecules reside at the oil-water interface.

nanometre-wide water channels but their growth might induce localized changes in the microstructure of the surrounding microemulsion. In this case, inorganic frameworks organized on longer-length scales might be synthesized by a process of 'chemical metamorphosis' within the inorganic-organic multi-component system.

The latter turned out to be the case when crystals were allowed to develop within bicontinuous microemulsions formed from the cationic surfactant, didodecyltrimethyl ammonium bromide (DDAB), supersaturated calcium phosphate solution, and tetradecane/hexadecane oils (Walsh et al 1994). We used long chain alkanes so that the oil phase could be frozen at temperatures ($+2^{\circ}\text{C}$) at which the supersaturated solution remained liquid. By doing so, we hoped to increase the rigidity and immobilize the underlying framework architecture of the reaction medium.

Scanning electron micrographs of the mineralized replicas extracted after two weeks showed the presence of highly reticulated microstructures of interconnecting needle-like ($0.2\text{--}1\text{ }\mu\text{m}$ in length) crystals of carbonated apatite (Fig. 3A). The microskeletal framework was constructed from an irregular array of single interconnecting apatite crystals, which were often over $1\text{ }\mu\text{m}$ in length and sometimes curved (Fig. 3B). The pore size distribution was heterogeneous, with some pores being mesoscopic ($<10\text{ nm}$) whilst others were $2\text{--}3\text{ }\mu\text{m}$ in dimension.

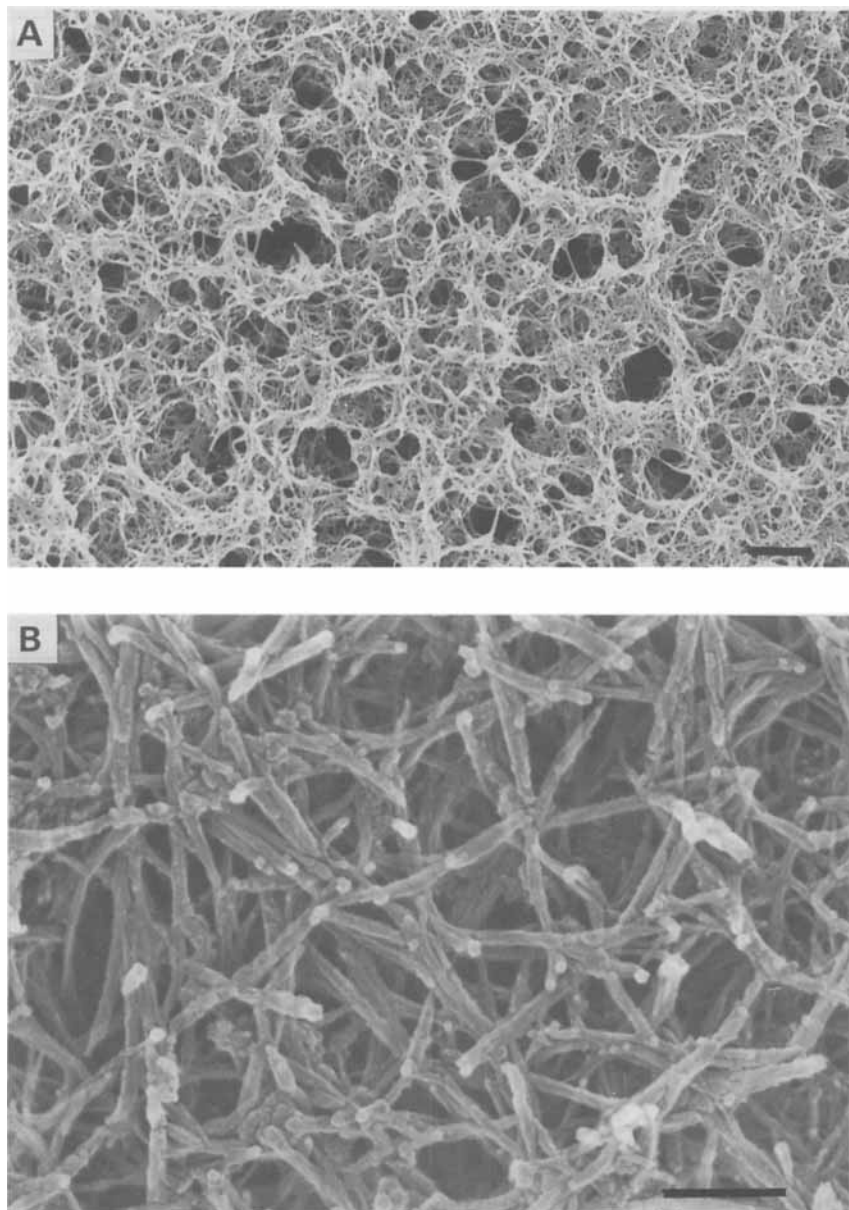


FIG. 3. Scanning electron micrographs of microstructural calcium phosphate formed in a bicontinuous microemulsion. (A) Low magnification. Bar = $2\text{ }\mu\text{m}$. (B) Higher magnification. Bar = $5\text{ }\mu\text{m}$.

The importance of microemulsion texture was investigated by studying inorganic architectures produced from supersaturated bicontinuous phases of the same composition but stored at lower or higher temperatures than the system described above. A preparation of the mixed oil system stored for 1 week at -25°C (that is, 31°C and 43°C below the melting points of tetradecane and hexadecane, respectively) produced highly elongated apatite crystals with lengths of 100–300 nm and widths within the range of 5–10 nm. Crystal growth did not proceed beyond this stage and no open-framed architectures were observed even after storage for several months. Conversely, the same supersaturated microemulsion stored unfrozen at $+25^{\circ}\text{C}$ produced intact reticulated structures composed of straight interconnected crystals together with areas of extensive aggregation.

A striking feature of the mature inorganic meshworks was that the wall diameters (*c.* 80 nm) were incommensurate with the 1 nm diameter water channels of the bicontinuous microemulsion phase. In fact, microstructures commensurate with these dimensions were only observed at the very early stages of precipitation (2–6 h at $+2^{\circ}\text{C}$) where filamentous strings of mineral particles dominate. The results suggested that nucleation of calcium phosphate is initially restricted to the interconnecting water conduits of the DDAB bicontinuous microemulsion but that subsequent growth within the frozen oil medium disrupts the microstructure possibly by adsorption of the surfactant molecules onto the developing crystal surfaces. Thus, the microskeletal form ‘evolves’ by reconstruction of the multi-component reaction medium with new patterns being expressed on longer-length scales. The process is superficially reminiscent of the way that enamel matures through resorption–redeposition processes.

The correspondence between microemulsion structure and the form adopted by *in situ* precipitation was highlighted by undertaking experiments in which the microemulsion had a very different microstructure. For example, calcium phosphates formed within microemulsions with compositions close to the edge of the bicontinuous phase domain did not form microskeletal structures but were spherical in morphology. Under these conditions, the microemulsion undergoes phase separation into a mixture of water and oil micelles, with the result that the mineral particles adopt the spherical morphology of individual supersaturated droplets.

Conclusions

Biom mineralization of enamel takes place through a sequence of processes that have general features capable of being mimicked in synthetic systems. I have described how concepts such as supramolecular preorganization, spatial patterning, interfacial molecular recognition, vectorial crystallization, pattern evolution and hierarchical assembly apply both to the formation of enamel and microskeletal calcium phosphate architectures deposited in organized bicontinuous microemulsions. The results of these preliminary studies suggest that biomimetic concepts could be useful in the fabrication of biomaterials with controlled porosity and microstructure.

Acknowledgement

I thank D. Walsh, University of Bath, for his important scientific contributions to the work on synthetic micro skeletal calcium phosphates.

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DISCUSSION

Zeichner-David: Just to play devil's advocate, why do you want to imitate nature when, as a chemist, you could create something that is better than nature?

Mann: Nature provides concepts that are usually a long way beyond what a chemist thinks about. For example, the ideas of replication, transcription and synergy don't come into the mind of a chemist. Although at the end of the day I would like to develop a material that has a better performance, currently I am working on the integration of these conceptual frameworks.

Snead: In your model you showed that as the tubes collapse they form interlocking pieces. Is it possible that the porosity in enamel, when it's subjected to various insults during enamel formation, is the result of the amelogenin nanospheres collapsing around the crystallites, so that extreme lateralization is observed rather than c -axial growth.

Mann: During cross-linking, calcium and phosphate are reacting and protons are released locally, so there are chemical gradients around the protein surface which could also induce what's happening around the crystal.

Fincham: Also, we know that the solubility of the amelogenins is sensitive to changes in local pH, and that pH differences affect the scale of the formation of the supramolecular structures. Therefore, local changes could have a profound effect on the matrix.

Simmer: Are the mineralized networks composed of smaller crystallites and if so, are those crystallites oriented?

Mann: Yes, they comprise a well-defined meshwork of smaller crystals. However, they are not oriented; rather, at this stage, it's a more of a spatial filling. In the sea urchin there is a similar kind of fenestrated cellular structure but this consists of a single crystal that grows over a large domain, rather than numerous crystals that are densely packed.

Slavkin: Does marine coral have a similar structure?

Mann: Yes, coral has a similar infrastructure, as have sponges, which are often made of silica but have a reticulated framework of individual rods that are held together.

Oldak: Iijima & Moriwaki (1991) have reported a study on the lengthwise and oriented growth of octacalcium phosphate on cation-selective membranes as a model for enamel formation. They have shown that it is possible to create c -axis-oriented nucleation of octacalcium phosphate when calcium ions are selectively introduced into the system. This is relevant to the situation in enamel when selective and vectorial calcium flux (by the ameloblasts) could direct apatite crystal growth without the need for a highly structured protein nucleator.

Elliott: You've addressed the confinement of the reaction space, but there are other mechanisms that control the habit of hydroxyapatite crystals. For example, adsorption of molecules on crystal faces can affect the growth mechanisms. Could you speculate on the role of these other mechanisms?

Mann: We have studied the effect of additives, particularly in the calcium carbonate system, rather than the phosphate system because the latter system is less sensitive in terms of habit formation. For example, longer hydroxyapatite crystals are observed in the presence of simple sugars such as glucose (Walsh et al 1993). We are not as interested in habit as we are in the development of form. There are numerous descriptions of habit formation in the literature, but these tend to address individual crystals, and we are trying to look at how we can make an inorganic material have 'complex form'.

Deutsch: Have you thought about demineralizing developing enamel in such a way as to retain its structure and then trying similar calcium carbonate experiments?

Mann: That's a good idea. However, the problem is that as soon as you do anything with a biological tissue then you are likely to disrupt the ultrastructure. It's easier to do in a synthetic system!

Robinson: The difference between what you've described and enamel is that the crystals are formed at the c -axis end in the wake of the retreating ameloblasts. We should be looking at the matrix structure immediately adjacent to that cell membrane. Once the crystal disposition is established here the crystals could grow in an almost formless gel.

Mann: Sheila Jones mentioned to me that the orientation of the cell wall is important for crystal growth. It seems as though the ultrastructure immediately beyond the cell wall gives rise to the vectorial relationship.

Diekwisch: Can you give us any advice on the nanosphere model of enamel crystal formation? The idea is that amelogenin nanospheres are present along the c -axis of

growing enamel crystals, but could the nanospheres actually come out of the ameloblasts and move along the crystal or would this destroy the crystallization process, i.e. might one expect to observe a zig-zag array if nanospheres are attached?

Mann: If I was studying these organic colloids, I would be looking for some evidence of an organized structure. As yet, there is no evidence that the amelogenins are organized in a periodic array. Low angle X-ray diffraction of hydrated samples is not a conventional way of looking at these structures but it may be one way of trying to find order in the 2–5 nm range. This has been done for a range of lamellar inorganic/organic materials, particularly in the field of silica chemistry.

Slavkin: Enamel formation is a dynamic process—it seems to involve different constituents at different stages. In the field of developmental biology there is a concept which explains a series of replacement events, such as fetal haemoglobin being replaced by adult haemoglobin. Is the making of an organ a series of replacement events? In terms of chemistry and bioengineering, are there now ‘nanotechnological’ methods that can be used to study replacement processes involved in the production of long ribbons of calcium hydroxyapatite?

Mann: It would be an extremely slow process because these techniques involve building a structure atom by atom. It is clear that in these microemulsion phases changes in structure and form accompany the chemical reaction, but to study what’s going on mechanistically would be difficult to do.

Slavkin: And it also isn’t clear which events are absolutely required for the process of apatite formation and which are involved in just keeping the cells alive.

Nanci: In mineralized sections through the enamel growth sites of well-preserved enamel organs and enamel one can see that the crystallites abut against the ameloblast cell membrane. Demineralized preparations show the same result. In this case, however, crystal ‘ghosts’ abut against the membrane. The enamel growth site is characterized by a ‘rarification’ of the matrix. One would, therefore, expect less protein at these sites. In fact, most of the anti-amelogenin antibodies we have used to date resulted in little immunoreactivity at enamel growth sites over a region of approximately 500 nm. This thickness of enamel corresponds to approximately 1 h of enamel production. If animals are treated with brefeldin A or cycloheximide to stop protein secretion, labelling with anti-amelogenin antibodies then extends up to the cell membrane, suggesting that proteins diffuse from deeper regions towards the enamel growth sites. The absence of immunolabelling for amelogenin at growth sites suggests that nanospheres are not present there and, hence, that they may not play a role in crystal elongation. Therefore, their action is likely to regulate growth in width. There are proteins at the enamel growth sites, and these are readily put in evidence by using a combination of protein secretion inhibitors and lectin cytochemistry (Nanci et al 1996). Certain antibodies to enamel proteins also show some labelling at these sites (Nanci et al 1996). The characteristics of the proteins at enamel growth sites suggest that they may correspond to the short-lived, sulfated and glycosylated protein described by Smith et al (1995).

Veis: How do the proteins jump across the growth site?

Nanci: The proteins do not jump across the growth site, although they may pass rapidly through it. Indeed, it has been shown that phosphoproteins of dentine are released in pre-dentine but accumulate only at the mineralization front (Weinstock & Leblond 1973). One possible reason for this behaviour may be their affinity for mineral. In the case of enamel, the preferential presence of certain proteins at growth sites may in itself serve to exclude amelogenins 'biophysically' from the area where crystals are actively elongating. Alternatively, amelogenins and non-amelogenin proteins may have different affinities for mineral. This paucity of amelogenins at growth sites would be consistent with the notion that they regulate the growth (in width) of crystals.

Robinson: There are problems with looking at the mineralization front using electron microscopy because the processing procedure involves the removal of water, which concentrates the mineral, and its replacement with low dielectric constant plastics. These procedures will push the mineral out of solution at the sites where that's going to happen in any case. Therefore, to say that the crystals are in a solid state at that specific micro-location is unwise.

Nanci: I agree that this is a problem. However, we have verified our results using rapid freezing and freeze substitution methods generally believed to preserve tissue close to the living condition (Nanci et al 1994), and enamel growth sites generally show a 'rarified' appearance. Whether the mineral here is in an amorphous or crystalline phase remains to be determined.

Snead: You have shown that amelogenin epitopes are present in the secretory vesicles right up to the plasma membrane, which represents a paradox to me. Where do these proteins go, since there is a 400 nm gap? It is possible that at this point there is immediate nanosphere assembly and a chaperone protein in the vesicles that prevents their immunodetection for 400 nm. This might simply be a matter of steric hindrance. That is, when the nanosphere forms, the only thing that is available is the C-terminal tail because it is externalized, so that within 1 h you find nothing but later you find the C-terminal pieces being chewed off.

Nanci: The enamel growth sites appear morphologically as 'rarified' areas that do not show significant immunoreactivity for amelogenin. These observations indicate that if nanosphere proteins are indeed present, they exist at a lower concentration than in deeper enamel.

Snead: I'm suggesting that the anti-peptide antibodies don't recognize their cognate epitope because the epitope is internalized, i.e. the amelogenin core is folded in on itself, masking the epitope.

Nanci: The antibodies we used are not peptide antibodies but polyclonal antibodies to purified proteins. Such polyclonal antibodies are expected to recognize several antigenic sites on a molecule. The majority of amelogenin antibodies we used gave similar results; that is, less labelling at enamel growth sites (Nanci et al 1996). Also, for immunolabelling we use the post-embedding colloidal gold method. With this method, epitopes are exposed at the surface of the section as the knife cuts through or fractures across the tissue. However, it cannot be ruled out that in some cases

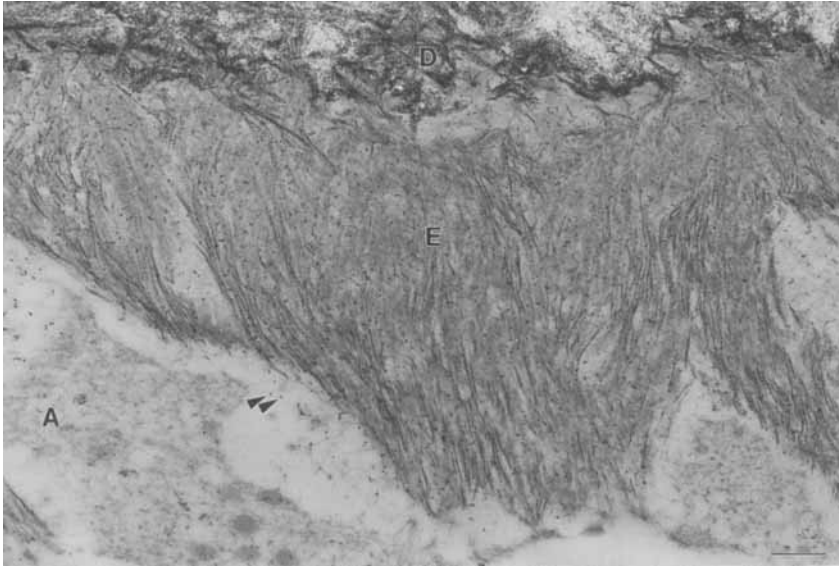


FIG. 1. In developing mouse molar enamel 5 nm immunogold particles are evenly distributed over the entire initial enamel layer. A, ameloblast; D, mantle dentine; E, initial enamel layer. Bar = 200 nm.

antigenic sites may not be exposed at the cut surface of the section (discussed in McKee & Nanci 1995).

Snead: So where does the protein go? You can detect the protein in the vesicle at the plasma membrane and then again at a distance of 400 nm. This 400 nm of lost proteins at the level of the protein represents a huge distance.

Nanci: There could be a gradient of protein across the growth site, with proteins accumulating in deeper enamel. The growth site is not 'empty', it is occupied by glycosylated and/or sulfated proteins, and perhaps also by some phosphorylated ones. These proteins may compete with amelogenin in the region of the growth site. Such competition may not occur beyond the growth site since the proteins present there have a short half-life (Smith et al 1995). Brefeldin A and cycloheximide studies have, indeed, confirmed that proteins characterizing the growth site turn over relatively rapidly (Nanci et al 1996). It is conceivable that as the enamel thickens proteins at growth sites are processed and 'prime' or 'make space' for amelogenins secreted by the cell or amelogenins flowing back from deeper areas of enamel.

Diekwisch: In my opinion, this spatial positioning of amelogenins around the developing enamel crystals is of extreme importance to provide a matrix for the growth of long parallel crystals. Our own immunogold studies have shown amelogenins evenly distributed over the entire initially developing enamel matrix (Fig. 1).

Nanci: When crystals are first initiated at the future dentine–enamel junction, it is likely that they are surrounded by both amelogenins and non-amelogenin proteins. However, as the enamel growth sites are established it seems that selected proteins become associated with the elongating extremity of crystallites, and this results in the ‘rarified’ morphology of enamel at growth sites. Indeed, Sasaki & Higashi (1983) have also shown using scanning electron microscopy that developing enamel surfaces have a ‘porous’ appearance.

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